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14. ABSTRACT

The focus of this proposal is to identify molecular markers in high grade prostate cancer (PCa) based on biopsies, especially from African Americans (AAs) who usually select radiation rather than radical prostatectomies. How these differ molecularly from European Americans (EAs) is of major interest. All IRBs and MTAs have been approved at participating sites. Dr. Gaston's laboratory has extracted DNA from 756 nitrocellulose blots from 63 patients including 40 AAs and racial admixtures have been determined on 12 of these cases; 2 out of 12 had significant differences from self declared race and DNA studies have been extended to paraffin blocks. Multiplex immunoasay pilot studies have been performed on serum, plasma and urine. Molecules of interest that are relatively elevated in PCa include EGF, sFAS2, CEA, G-CSF, il-13, and MIP-1β. Molecules that are relatively decreased include free PSA, He-4, il-1a, il-1b, and TGFβ. Work with Dr. Clayton Yates has identified microRNAs that are differentially expressed in PCa and show racial differences including microRNA-152 which correlated with aggressive features. Also, epigenetic field effects induced by PCa have been identified.

15. SUBJECT TERMS
Prostate cancer, molecular markers, multiplex immunoassays, racial differences

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INTRODUCTION

Adenocarcinomas of the prostate (PCa) that occur in African Americans (AAs) are reported to have a worse prognosis than PCas occurring in European Americans (EAs); however, the molecular causes as to this increased aggressiveness of PCas in AAs has not been identified or studied adequately. We hypothesize that the different molecular characteristics of PCas could be used in determining aggressiveness or clinical outcome (prognosis) based on race and/or ultimately for targeting by novel therapies, biomarkers and/or their signal transduction pathways.

Our first approach is to identify biomarkers in PCa that are expressed differentially based on analysis of PCas from AAs compared to those from EAs and to evaluate those that are more likely to be associated with aggressive features of PCas. Initially, proteins, mRNA and DNA will be extracted from nitrocellulose blots of biopsies of the prostate which are being collected at UAB and Urology Centers of Alabama (UCA). Racial admixture studies (SNPs and mitrochondrial DNA) will be used to modify self declared race. Then, the blots of PCa of the prostate will be analyzed by Affymetrix assays performed to identify genes that vary with race in PCa and that are associated with aggressive features (e.g., Gleason scores ≥ 7). These results will be confirmed in an independent set of samples by RT-Q-PCR. Similarly, proteins associated with racial differences and/or aggressiveness will be identified by liquid chromatography mass spectrometry (LCMS) and by multiplex immunoassays (Luminex technology). These proteomic assays will be performed on serum (or plasma) and urine. Separately, samples from 150 AAs and 150 EAs who are undergoing evaluation for prostate diseases and are age matched ($\pm$ 5 years) will be analyzed for racial admixture to characterize racial characteristics of the Alabama male population with diseases of the prostate.

After these initial discovery studies, an independent set of samples from biopsies of the prostate will be used to verify molecular features that have been identified to be expressed differentially in AAs vs. EAs and that also are associated with aggressiveness in AAs. Molecular features that meet these criteria will be analyzed by RT-Q-PCR, multiplex immunoassays, and mass spectrometry to confirm the preliminary results.

BODY

Administrative: In 2013, multiple administrative issues were successfully resolved; some of these had delayed components of our studies of racial differences in the aggressiveness of prostate cancers. These administrative delays were associated with Dr. Gaston's move to Tufts University (TU) and the associated changes that were necessary to move forward with specific areas of the grants. Specifically, all IRBs at UAB, TU and Urology Centers of Alabama were approved and/or renewed. The approvals of the IRB at TU permitted materials transfer agreements (MTAs) to be developed and approved between UAB and TU. The grants of Dr. Gaston and Dr. Grizzle were extended at no cost until 6/30/2015.

Dr. Grizzle participated in several DOD initiatives. Specifically, he continues to be a consultant to the DOD Prostate Cancer Tissue Repository and he participated in the symposium "Validating Tissue Biomarkers in Primary and Metastatic Prostate Cancer" organized by this group in Orlando, Florida in February 2013. In addition, he is a participating member of the External Advisory Committee of the DOD Repository of Lung Cancer.

Also of note, Dr. Gaston has been appointed as Scientific Director of the Tufts Medical Center Tissue Repository beginning October 2013. She was a speaker at the Cambridge Healthtech Institute Annual Sample Prep and Target Enrichment in Molecular Diagnostics conference in April 2013 and participated in the Institute for International Research Biorepositories and Sample Management Conference in September 2013.

Specific Progress and Results: Racial Differences in Epigenetics and Post-Transcriptional Regulation of Proteins: Enough studies are now available to indicate that many features of cancer are not caused by the germ line genomic sequences. Two areas upon which we are focusing are the post-transcriptional regulation of signals that affect the development and progression of cancer and epigenetic regulation of gene expression in PCa (1, 2). One focus in these studies is on small RNAs that regulate mRNAs either to increase or to decrease specific proteins. Of these, microRNAs (miRs) are the best characterized (1-3). Initially, our laboratories are collaborating with the laboratory of Dr. Clayton Yates at Tuskegee University in characterizing racial differences in microRNAs. To date, we have microdissected 34 paired samples (34 cancers and 34 uninvolved) of PCa from AAs and 34 similarly paired samples of PCa from EAs. RNA (mRNA and microRNA) has been extracted from these samples and provided to Dr. Yates' laboratory who screened these to identify miRs which are expressed differentially in AA versus EA cases. To date, 39 of these cases have been analyzed as a learning set (one case has to be repeated). Of interest, 11 miRs were differentially expressed between PCa

and uninvolved samples with p values adjusted for multiple comparisons ranging from 0.009 to 3.5x e-05. These are listed in Table 1 (4).

Of these miRs, we initially focused on miR-152 which is decreased in most specimens 14/20 from AAs and 13/19 in EAs (Figure 1). The decreased levels of miR-152 was found to be correlated with increased metastasis and biochemical recurrence. These data have been combined in studies of cell lines and additional PCa primary and metastatic tumors using in silico analysis and laboratory studies at Tuskegee of selected PCa cell lines. MiR-152 was found to bind and down regulate DNA (cytosine 5) methyltransferase 1 (DNMT1) and there appears to be a feedback loop between the expression of DNMT1 and miR-152. These results have been incorporated into a manuscript "Genome-wide microRNA profiling of novel African American and Caucasian prostate cancer cell lines reveals that miR-152 is silenced by DNA methylation" by Theodore et. al. which has been submitted for review (4).

Prostate Cancer Tissue Biomarkers: We have prospectively enrolled 63 study subjects for our gene expression studies in which we utilize innovative tissue print techniques to obtain snap-frozen "touch prep" samples from the diagnostic prostate biopsy cores. Based on self-identified ancestry, 40 of these men (63%) are Black/AA, 22 (35%) are White/EA and 1 (2%) are "other". As of September 2013, prostate biopsy tissue samples from all 63 study subjects have been transferred from Dr. Grizzle's laboratory to Dr. Gaston's laboratory for analysis. These samples include 12 nitrocellulose blots on each of 21 AA cases collected from Urology Centers of Alabama (total of 252 blots) and 12 blots per case on 19 AA cases, 22 EA cases and 1 "other ancestry" case collected at UAB (total of 504 blots). The age distribution of these 63 subjects is similar to that observed in other prostate biopsy cohorts in the USA. The proportion of subjects with benign biopsy findings, low grade prostate cancer and high grade prostate cancer is similar in our self identified AA and EA groups.

Detailed biopsy pathology reports are available for all 63 study subjects enrolled to date. As expected, approximately 55-60% of our prospectively enrolled cohort was diagnosed with cancer on biopsy and the proportion of subjects who were diagnosed with low grade (Gleason 3+3) and high grade (Gleason 7 or more) cancer is similar in each racial group. Based on the prostate biopsy pathology reports, 10 of our subjects (2 EA and 8 AA) would be considered good candidates for Active Surveillance as opposed to immediate treatment. Nationally, there are concerns that the criteria being developed for selecting patients for Active Surveillance may not be appropriate for AA men, given their higher risk for aggressive prostate cancer. Based on our findings that epigenetic DNA methylation markers may be useful for identifying patients with occult high grade prostate cancer that has been missed due to biopsy sampling error (see section "*Related Studies to Support This Grant")*, we plan to incorporate a subset of our UAB and UCA study subjects into a pilot study comparing methylation marker field effects in AA and EA prostate biopsy patients.

One of our primary project goals is to obtain gene expression data from high grade prostate cancers that are not treated surgically and for that reason are not available in conventional tissue repositories that depend upon radical prostatectomy specimens. We have collected biopsy tissue prints from both AA and EA subjects that clearly represent this high risk patient population. Figure 1 shows a summary diagram of the biopsy report of one such study subject, a 71 year old AA diagnosed with a Gleason 10 grade prostate cancer involving most of the left side of the gland. Our biopsy tissue print samples provide a unique source of high quality RNA and DNA for molecular analysis of such high risk prostate cancer patients who would not have tissue available from radical prostatectomies. We are utilizing this resource for both transcriptional and epigenetic biomarker analysis.

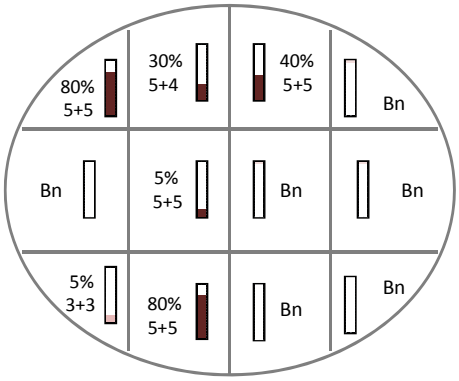

Figure 1. Diagrammatic summary of biopsy pathology report for study subject P455 (self identified AA, age 71 at biopsy). Consistent with current clinical practice, this individual's large, very high grade prostate cancer was treated with hormonal therapy and radiation. If we had not collected biopsy tissue prints for this case, frozen tissues would not have been available for research because all biopsies would have been processed into paraffin blocks and no tissue would have been available from a radical prostatectomy.

Dr. Gaston's laboratory has completed DNA purification for ancestry genotyping for all 63 subjects enrolled in the biopsy tissue print collection study. Because the tissue prints are snap frozen samples, analysis of DNA ancestry markers is straightforward. DNA ancestry marker analysis by autosomal SNP genotyping is in progress in Dr. Rick Kittles laboratory in Chicago and genotyping has been completed for 12 of these 63 subjects. A pilot analysis of mitochondrial ancestry informative (haplogroup) sequences in which PCR amplicons of HSVIII segments were obtained from biopsy tissue print DNA and sent to SeqWright Laboratories for sequencing has also been successfully completed.

The ancestry informative marker panel utilized by Dr. Kittles laboratory provides estimates the % autosomal ancestry from West African, European and Native American lineages. For the subjects for whom genotyping is completed, we observe that 10 of the 12 show ancestry markers highly consistent with self identified ancestry (>85% match to the self-identified ancestry), 1 of the 12 showed significant ancestry admixture and the individual self-identified as "other" could be assigned to an ancestry group (>93% match). This data is critical as we move ahead to assign our subjects to specific groups for the mRNA profiling studies.

SNP genotyping is also in progress for the FFPE specimens that will be used for our admixture prevalence analysis. It is widely recognized that DNA recovered from FFPE tissues can be more difficult to analyze due to degradation and we have undertaken careful preliminary studies to evaluate the quality of the DNA recovered from our DNA specimens. We are pleased to report that 100% of our pilot test FFPE DNA samples were fully genotyped by the Kittles laboratory. This marks an important project milestone and allows us to move forward with the full set of prevalence study samples.

Multiplex Immunoassays: Multiplex immunoassays permit concomitant assays of up to 100 individual antigens on one specimen, and hence, represent an excellent approach to screening for potential protein biomarkers in prostate cancer. Of special interest in multiplex assays are biomarkers that are associated with aggressiveness and vary with race.

We became interested in the roles of cytokines in prostate cancer based upon the observation of Veltri et. al. (5, 6) that higher serum levels of interleukin 8 are more useful than PSA in identifying cases of prostate cancer. This was of special interest because UAB had previously identified that interleukin 8 (il-8) and il-6 were increased in patients with pancreatic cancer. Thus, we began by evaluating several commercial multiplex immunoassays of serum from patients with and without prostate cancer. Our initial studies of two commercial kits (Millipore) measured multiple cytokines (Cytokine 27 antigen) panel and tumor markers (16 antigens). However, many of the measurements of specific cytokines in serum and/or plasma from patients with and without PCa were below levels of detection. Unfortunately, we were unable to obtain adequate support from Millipore as to the sensitivity of the kits so we shifted to multiplex immunoassay kits developed by BioRad which is the manufacturer of our multiplex Luminex immunoassay system (BioPlex) and which maintains this system. BioRad has proven to be much more responsive to the issues raised as to their assays.

In this report period, we worked with BioRad 1) to evaluate how our multiplex system functions. This initial evaluation identified a problem with our automated plate washer which has been repaired and the software used in analysis of the BioPlex assays was updated and upgraded. 2) After repairs, the BioRad assays kits were evaluated on prior samples that have been run on the system to ensure that there was adequate sensitivity of these kits. These assays were run in conjunction with the on-site training at UAB of a new BioRad engineer in multiplex-Luminex technology by the Chief Engineer of BioRad and the Technical Representative of BioRad for UAB. Specifically, in these assays, results in serum and plasma pairs from the same patient were evaluated as well as some samples previously analyzed. The Chief Engineer and Technical Representative were both very pleased with the final function of our instrumentation and new software and the knowledge and performance of our technologist who performed the assay; 3) performed and analyzed multiplex assays on urines from patients with PCa.

Each individual cytokine varies as to its levels as measured in serum versus plasma. Of importance, measurements of levels in different bodily fluids (e.g., serum and plasma) can be mixed in one BioPlex assay depending on the range of concentrations in each bodily fluid. Thus, il-8 levels in serum can be measured together with il-6 levels in plasma in the same BioPlex assay. Nevertheless, this is not an efficient approach to multiplex assays because all cytokines are not measured in one sample and it would be preferable to measure all molecules of interest in a single sample of bodily fluid. Therefore, we evaluated the 27 plex cytokine assay in samples of urine from patients with and without a diagnosis of PCa. The 27 plex assay from BioRad was run on matched serum and plasma samples from 10 patients with PCa and 6 patients

without PCa, and separately on urine samples from 20 patients with elevated PSA but without PCa on biopsy and 15 patients with elevated PSA and biopsy proven PCa.

The population being evaluated by Luminex (i.e., BioPlex) technology to identify racial differences in PCa is challenging in that only patients being evaluated for PCa are included. Thus, there is no normal group because such cases are too easy a comparison and would not represent a true, real medical sample comparison, i.e., usually normal individuals without elevated PSA and/or symptoms are not elevated for prostate cancer. Most of the patients have elevated levels of PSA and are divided into two primary groups – patients with biopsy proven PCa and patients in which PCa was not identified on biopsy. Also, excluded from both groups are patients with other malignancies and/or with high grade PIN on biopsy but no identified invasive PCa. Patients with PIN are not at much greater risk for invasive PCa. Thus, in these studies, the major problem is that some patients with elevated PSA with no tumor on initial biopsy actually may have PCa that was missed on biopsy. Thus, when samples of bodily fluids are obtained, the PCa group is well defined but the group with elevated PSA but no tumor on biopsy remains not well characterized as to the absence of PCa, because some cases of PCa are relatively indolent as to their speed of progression and are difficult to detect and because some patients evaluated for PCa may have other undiagnosed tumors (e.g., one had aggressive a lymphoma diagnosed subsequently).

These studies demonstrated the following generalizations:

1. Most cytokine related molecules are expressed at higher levels in plasma than in serum. These include G-CSF and il-13. However, some cytokine related molecules are expressed at higher levels in serum, e.g., MIP-1β.
2. In general, urine values of cytokine molecules are lower than serum or plasma values.

There are multiple issues that have been raised by our multiplex immunoassay studies of cytokine related molecules:

1. The initial biopsy performed immediately following obtaining the blood and urine samples sometimes missed the PCa which was identified subsequently on further evaluation. We are now reviewing the patient histories of all samples of bodily fluids of the patients diagnosed as not having cancer.
2. There is a large variability of levels of cytokine related molecules in both the PCa and non-PCa samples. Some of the variability in PCa cases may be due to the extent of disease, and in the PCa and non-PCA cases to the extent of inflammation.

In the study of serum versus plasma using the 27 plex assays, we identified G-CSF, il-13 and MIP-1β as having elevated levels in both plasma and serum of cancer cases.

Even with this problem with the definition of "controls," based on prior studies, three other molecules seem to be elevated in the PCa group and these elevations are consistent with elevations seen in other tumors. These are epidermal growth factor (EGF x 1.9 in tumors), solable Fas ligand (sFASL x 4.6 in tumors), and carcinoembryonic antigen (CEA x 1.3 in tumors).

Based upon the BioRad and Millipore assays, sFASL, EGF, CEA, il-13, G-CSF and MIP-1β are potential molecules of interest based upon increased levels in cases of cancer. Alternatively, though less attractive are free PSA, He4, il-1a, il-1b and TGFβ which are relatively increased in patients without PCa. We will repeat results and set up an assay based on race to clarify further molecules of interest.

In the last year, we have been working with Dr. James Mobley and our proteomics facility in obtaining new cutting edge mass spectrometry equipment. This equipment has been installed, tested and is ready to run specimens of this project. Specifically, we will be using this latest instrumentation in mass spectrometry to survey the global proteome extracted from the clinical prostate specimens (frozen, FFPE, and bodily fluids). This includes a new complete 1260 nano-HPLC system with autosampler from Agilent in-line with a Hybrid Orbitrap Velos Pro mass spectrometry system from Thermo. The configuration includes a 20cm x 75u I.D. column that separates trypsin peptide digests using an optimized 3 hour gradient. This generally produces well over 1000 proteins with a single injection from as little as 500ng of protein extracts from tissue specimens. With this combination we can ID more unique proteins within a single experiment than any other mass spectrometer on the market. The informatics used to drive the analysis include a newly purchased top of the line high speed server from Dell, tied to a 1000 node cluster. Unique programs used for quantitative analysis include Refiner MS from Genedata, which allows the lining up of all 1D LCMS runs by 2D time and mass tags (AMT), whereby each peptide is quantified as the area under the curve, and normalized across each independent run, to then be compared across an entire set of samples.

Studies Related Studies to Support This Grant:

In our quarterly progress reports for this year, we noted an opportunity to collaborate with Dr. Clayton Yates at Tuskegee University in an evaluation of racial differences in selected microRNAs in prostate cancer. This collaboration, as discussed, has been very successful. This collaboration will particularly focus on patients with high grade prostate cancer, to take advantage of our access to samples from patients from whom no tissue is available from radical prostatectomies. After consultation with the technical support staff at two different companies (Qiagen and Zymo), we are testing an updated version of two different DNA/RNA nucleic acid preparation kits that should allow for better recovery of microRNAs in the purified RNA prepared from our biopsy tissue print samples.

In our quarterly progress reports for this year we also described a study initially supported in part by the NCI Early Detection Research Network (EDRN) in which we have used tissue prints of diagnostic prostate biopsies to determine if the field effects generated by cancer-associated DNA methylation might serve as a useful test for the presence of occult prostate cancer in the tissues adjacent to the biopsy core. Many studies have shown that tumor-associated changes in DNA methylation can act as biomarkers for the presence of prostate cancer. We have identified three DNA hypermethylation markers (GSTPi, APC and RASSF1A) with field effects that extend far enough around the histological boundary of a prostate cancer focus to be detected in a biopsy core several millimeters away. Importantly, we found that both the magnitude and the extent of field-effect hypermethylation are sensitive to the grade of the adjacent prostate cancer. Dr. Gaston's laboratory has now extended these studies with an analysis of prostate biopsies from more than 90 subjects to evaluate a methylation marker "signature" that is sensitive to the presence of high grade prostate cancer in adjacent tissues (cancer that was missed due to biopsy sampling error). These data are summarized in Table 3 and were presented at the February 2013 ASCO Genitourinary Symposium (the published abstract from this meeting is attached to this annual progress report).

Future Directions:

In addition to completing the plan of work for this DOD project, we have made a commitment to collecting and annotating our set of study samples as a resource for future health disparities research. It should be noted that all of our study samples will be genotyped for ancestry informative markers in an IRB approved manner. Our prospectively collected biopsy tissue print samples, which produce high quality RNA and DNA from the entire clinical range of biopsy patients (men diagnosed with no cancer, active surveillance and high risk cancer) are being processed to support mRNA, miRNA and DNA analysis of both tumor and adjacent uninvolved tissues. In this next quarter, we will develop a prototype database that can track each of the biopsy tissue prints (currently 756 from 63 study subjects) and our genotyped FFPE prostate cancer patient specimens to maximize their use for this and future projects.

Post-transcriptional and Epigenetic Regulation: We plan to analyze, together with Dr. Yates' laboratory, up to 100 cases of PCa from AAs versus EAs for differential expression of microRNAs based upon race. Each case will be macrodissected. Each of the miRs that have different expression in AAs will be analyzed independently for its gene targets and for the effects of changing the levels of the miR on aggressive features (e.g., control of cellular death, proliferation, cellular mobility and invasion). Of interest, miR-152 was found to be downregulated via methylation of its promoter and to have a feedback regulatory loop with its mRNA target, DNMT1, a methyltransferase. Features of aggressiveness also were correlated inversely with the phenotypic expression of miR-152. The next miR that will be explored will be miR-132. Epigenetic regulation of all mRNAs also is being evaluated.

Study of Racial Admixtures in Patients Evaluated for Prostate Diseases: Because of new techniques in extraction of DNA and RNA from paraffin sections, new paraffin sections had to be cut from the 300 cases of prostate tissues (150 AA and 150 EA) from patients who were elevated and/or treated for prostate diseases. This was necessary to reduce the paraffin being carried over into assays when paraffin sections were cut from blocks and put into microfuge tubes rather than mounted on glass slides. Recutting paraffin sections (five 12μ sections per block) and mounting them on microscope slides has been completed for over 200 cases of the 300 cases needed. The remainder of cases will be cut in the next quarter. Dr. Kittles' laboratory will complete the racial admixture studies on the remaining 51 patients from whom nitrocellulose blots have been obtained (63) to date and will begin analyzing the DNA from FFPE tissues.

KEY RESEARCH ACCOMPLLISHMENTS

- Identified microRNAs that were differentially expressed in prostate cancer from AAs and EAs (table 1). Demonstrated that miR-152 is epigenetically downregulated by methylation and its target, DNA methyltransferase 1 (DNMT1), has a feedback loop with miR-152. Decreased expression of miR-152 is associated with aggressive cases of PCa (e.g., metastatic and recurrent as well as increased invasion and proliferation).
- Completed a pilot study of the expression of 27 cytokines in urine specimens from 35 patients with and without PCa and a comparison of 27 cytokines as to their differential expression in serum versus plasma in 16 patients with and without PCa.
- Macrodissected paraffin blocks and extracted RNA and DNA from paraffin sections from 14 pairs of PCa and of matching uninvolved prostate from AA patients and 14 similar pairs from EA patients. Provided these RNAs to Dr. Yates for studies of microRNA and mRNA.
- Recut paraffin tissue sections for extraction of DNA from 200 cases (100 AA and 100 EA) for admixtures in patients being evaluated for prostate diseases.
- To date, 756 nitrocellulose blots have been obtained from 63 men undergoing biopsies (12 per patient). The DNA on these patients has been extracted. The racial admixture based on DNA for 12 of the 63 cases has been analyzed. Out of the 12 specimens analyzed, 1 unknown was assigned to a racial group and 1AA case had a significant EA component.

REPORTABLE OUTCOMES

- All administrative issues have been resolved. All IRB approvals and/or renewals have been completed for UAB, TU and UCA. Material transfer agreements developed and approved between UAB and TU.
- Preliminary study of racial differences in microRNAs of PCa completed. Manuscript submitted.
- Several review manuscripts associated with this grant are published and/or in press (listed in body and included in appendix).
- Dr. Gaston presented at the 2013 ASCO Genitourinary Symposium (attached) discussing the field effects due to methylation of DNA promoters based on biopsies.

Problems and Challenges: The major problems during this year were administrative. These included IRB approvals which have been obtained and the development of material transfer agreements which are complete. Recent changes in health care funding have reduced the number of biopsies performed at UCA outpatient site and biopsies from African Americans are now being performed at UCA hospital facilities. Also, a research nurse was lost at the UCA outpatient facility and a new research nurse had to be trained. A new research nurse has been trained and biopsies are again being obtained, but the sites where biopsies are now available from African Americans have now shifted to less convenient and more widely dispersed sites. Nevertheless, we do not expect problems in obtaining blots of biopsies from AA patients for this study.

CONCLUSION

Our initial administrative issues have been resolved.

The study of racial differences in microRNAs in prostate cancer including the association of miRs with aggressive subtypes of PCa has proven to be an important and successful area of study. Of these, miR-152 is associated with aggressive features of PCa. Of interest, specific miRs that are differentially decreased in PCa may be epigenetically down regulated via methylation.

The study of cytokines and tumor biomarkers using multiplex immunoassays has proven challenging due to difficulties in the identification of true controls. Cytokines have been quite variable in controls probably because some have unidentified PCa, another crytogenic neoplastic process, or extensive inflammation, all of which may increase specific cytokines. In addition, there seems to be inconsistencies with measurements of specific cytokines using commercial assay kits from different companies (e.g., Millipore vs. BioRad vs. R&D). Our instrumentation with upgraded software is now functioning optimally and several putative biomarkers that are relatively increased or decreased in PCa have been identified and are being evaluated in other sample sets for which the clinical histories of each control patient is being reevaluated for the immediate period after samples were obtained. These biomarkers include EGF, sFASL, CEA, il-13, G-CSF and MIP-1β.

DNA and RNA has been extracted from 63 patients and racial admixtures has been determined on 12 of these patients in the Kittles laboratory. The racial admixture study was significantly different from declared race in 2 of these 12 patients.

Our methodology has demonstrated that DNA can be extracted from formalin fixed paraffin embedded tissues. In our pilot study, 100% of our cases yielded DNA which can be used in genotyping. DNA is now being extracted from the paraffin blocks of biopsies and other prostate tissues; the DNA and protein are being genotyped and the proteome is being analyzed by mass spectrometry (8).

In summary, our team of investigators is successfully working on all our specific aims in this project.

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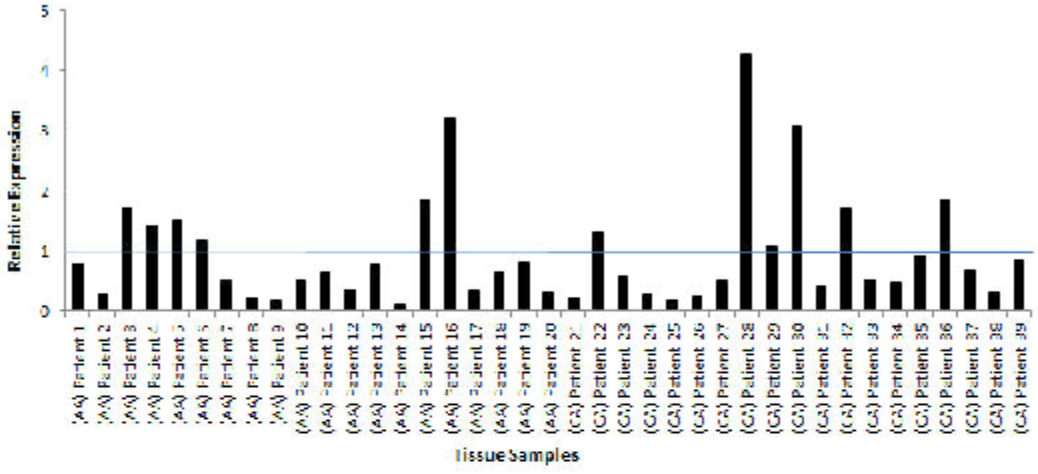
SUPPORTING DATA

Table 1

MicroRNA	(Adjusted) p-value ↑
hsa-miR-363	3.43e-05
hsa-miR-132	0.000354
hsa-miR-376b	0.00188
hsa-miR-410	0.00291
hsa-miR-152	0.00471
hsa-miR-189	0.00498
hsa-miR110	0.00514
hsa-miR-27b	0.00657
hsa-miR-519c	0.0071
hsa-miR-520h	0.00774
hsa-miR-27a	0.00837

Fig 2

a.


Relative Expression
Tissue Samples

b.

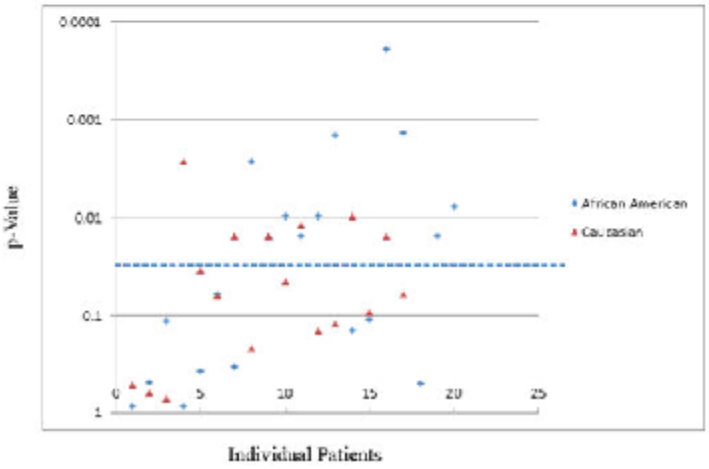

p-Value
African American
Caucasian
Individual Patients

Fig 3

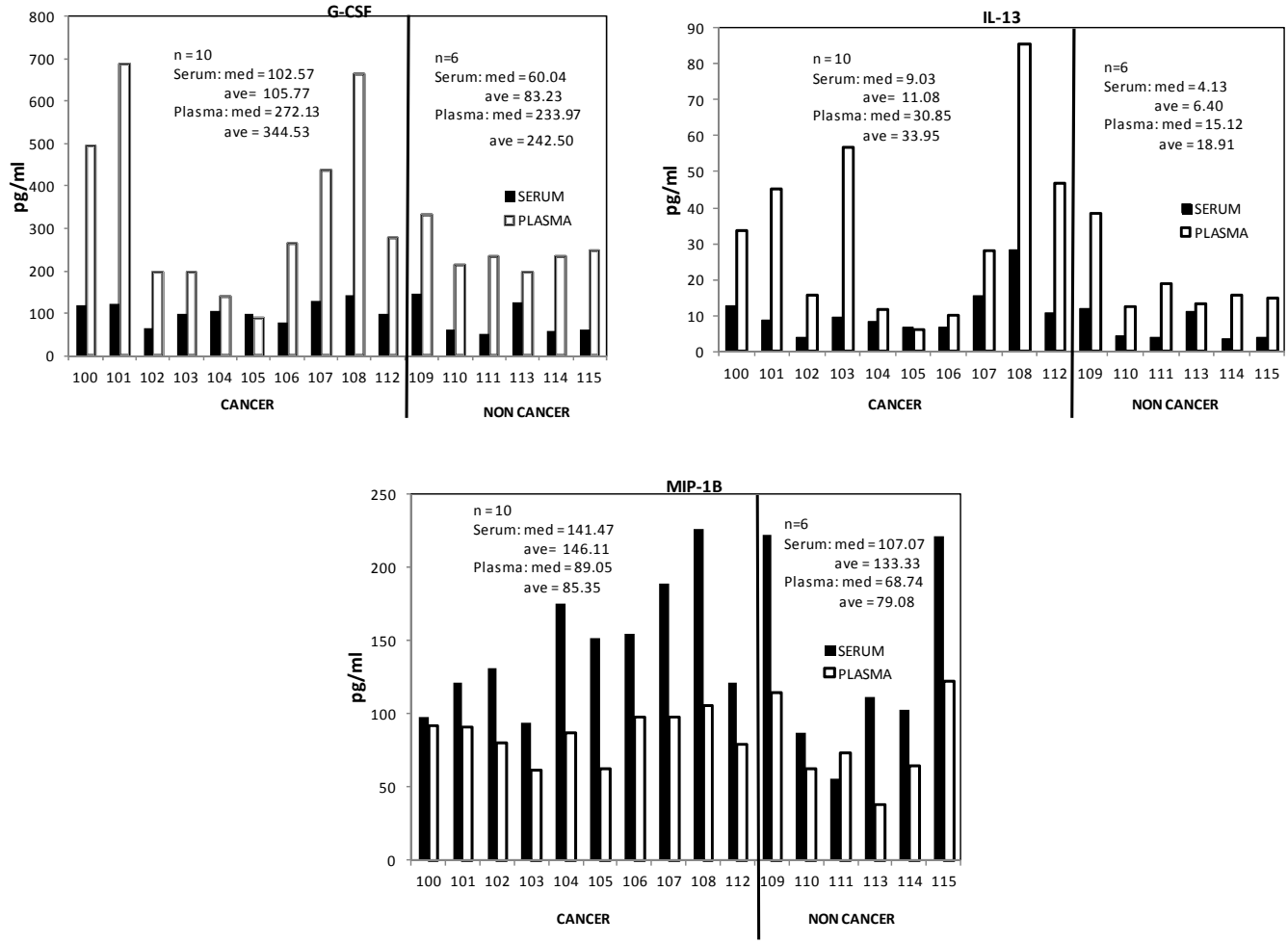

G-CSF
n = 10
Serum: med = 102.57
ave= 105.77
Plasma: med = 272.13
ave = 344.53
n=6
Serum: med = 60.04
ave = 83.23
Plasma: med = 233.97
ave = 242.50
SERUM
PLASMA
pg/ml
CANCER
NON CANCER
IL-13
n = 10
Serum: med = 9.03
ave= 11.08
Plasma: med = 30.85
ave = 33.95
n=6
Serum: med = 4.13
ave = 6.40
Plasma: med = 15.12
ave = 18.91
MIP-1B
n = 10
Serum: med = 141.47
ave= 146.11
Plasma: med = 89.05
ave = 85.35
n=6
Serum: med = 107.07
ave = 133.33
Plasma: med = 68.74
ave = 79.08

Table 2

Epigenetic Methylation in Benign Biopsy Cores:
No Cancer Controls vs Cases with Cancer Elsewhere in the Gland

All 3 Markers	Below Test Cut-offs for All 3 Markers		Above Test Cut-offs Any Cores, Any Marker		Above Test Cut-offs 1-2 Cores, Any Marker		Above Test Cut-offs 3+ Cores, Any Marker	
	N	%	N	%	N	%	N	%
Group 1 (N = 20)	11	55.0%	9	45.0%	7	35.0%	2	10.0%
Group 2 (N = 34)	10	29.4%	24	70.5%	16	47.0%	8	23.5%
Group 3 (N = 21)	4	19.0%	17	81.0%	4	19.0%	13	62.0%

APC	Below Test Cut-off APC		APC Above Test Cut-off Any Cores		APC Above Test Cut-off 1-2 Cores		APC Above Test Cut-off 3+ Cores	
	N	%	N	%	N	%	N	%
Group 1 (N = 20)	16	80.0%	4	20.0%	4	20.0%	0	0.0%
Group 2 (N = 34)	18	53.0%	16	47.0%	9	26.5%	7	20.6%
Group 3 (N = 21)	5	23.8%	16	76.2%	5	23.8%	11	52.4%

GSTPi	Below Test Cut-off GSTPi		GSTPi Above Test Cut-off Any Cores		GSTPi Above Test Cut-off 1-2 Cores		GSTPi Above Test Cut-off 3+ Cores	
	N	%	N	%	N	%	N	%
Group 1 (N = 20)	18	90.0%	2	10.0%	2	10.0%	0	0.0%
Group 2 (N = 34)	24	70.6%	10	29.4%	8	23.5%	2	5.9%
Group 3 (N = 21)	13	62.0%	8	38.0%	4	19.0%	4	19.0%

RASSF1	Below Test Cut-off RASSF1		RASSF1 Above Test Cut-off Any Cores		RASSF1 Above Test Cut-off 1-2 Cores		RASSF1 Above Test Cut-off 3+ Cores	
	N	%	N	%	N	%	N	%
Group 1 (N = 20)	12	60.0%	8	40.0%	6	30.0%	2	10.0%
Group 2 (N = 34)	15	44.1%	19	55.9%	13	38.2%	6	17.7%
Group 3 (N = 21)	6	28.6%	15	71.4%	6	28.6%	9	42.8%

p-values for the Chi square test for trends in proportions were calculated to compare the proportion of marker-positive subjects (defined as any histo-benign core being marker-positive) in the 3 study groups. These data are highlighted in yellow on the table to the left.

When we use all 3 markers, we see a consistent increase in the proportion of field effect positive subjects from group 1, group 2 and group 3. (from 45%, to 70.5% to 81%)

- For all three markers: p = 0.015

For each individual marker, this increase in the prevalence of field effects with Gleason grade is most pronounced for APC.

- For APC: p = 0.00031
- For GSTP1: p = 0.043
- For RASSF1: p = 0.043

Tissue prints were prospectively collected from biopsy cores from study subjects undergoing diagnostic prostate biopsies. DNA from all 12 cores was analyzed for the DNA hypermethylation markers glutathione-S-transferase pi 1 (GSTP1), adenomatous polyposis coli (APC) and ras association domain family member 1 (RASSF1). Levels of hypermethylation are reported relative to a reference gene, beta actin (ACTB).

APPENDICES

Cumulative Publications Resulting from this DOD Grant:

A. Zhang HG, **Grizzle WE**. The effects of exosomes and related vesicles on cancer development, progression and dissemination. *In: Emerging Concepts of Tumor Exosome-Mediated Cell-Cell Communication*, (Eds HG Zhang), Springer Science. 2012;107-129.
B. McNally LR, Manne U, **Grizzle WE**. Post-transcriptional processing of genetic information and its relation to cancer. Biotech Histochem 2013; 88(7):365-72. **(This was in press during the last report. A final version of the published manuscript is included).**
C. Srivastava S., **Grizzle WE**. Biomarkers and the genetics of early neoplastic lesions. Cancer Biomark 2011;(9)(1-6):41-64. NIHMSID:NIHMS402044.
D. **Grizzle WE**, Srivastava S, Manne U. The biology of incipient, pre-invasive or intraepithelial neoplasia. Cancer Biomark 2011;9(1-6):21-39. NIHMSID:NIHMS402045.
E. **Grizzle WE**, Srivastava S, Manne U. Translational pathology of neoplasia. Cancer Biomark 2011;9(1-6):7-20. NIHMSID: NIHMS402047.
F. **Gaston SM,** Guerra AL, Grooteclaes M, Renard, I, Kearney MC, Bigley J and Kearney GP. Gene Methylation Biomarker Analysis of Prostate Biopsies from Men with 1 of 12 Cores Positive for Cancer: Greater Methylation Prevalence and Extent in Gleason 7 than Gleason 6 Cancer. American Urological Association Annual Meeting, Washington DC; May 2011.
G. **Gaston SM**, Kearney GP, **Grizzle WE**. Prostate biopsy tissue print technologies; a practical and innovative approach to overcoming racial disparities in the datasets used for prostate cancer biomarker development. Presented at the AACR Cancer Health Disparities Meeting, Washington, D.C., September 18, 2011.

Publications since last report (**included in Appendix**):

H. Otali D, He Q, Stockard CR, **Grizzle WE**. Preservation of immunorecognition by transferring cells from 10% neutral buffered formalin to 70% ethanol. Biotech Histochem 2013; 88(3-4):170-80. NIHMSID:NIHMS493088.
I. Jones J, **Grizzle W**, Wang H, Yates C. MicroRNAs that affect prostate cancer: emphasis on prostate cancer in African Americans. Biotech Histochem 2013; 88(7):410-24.
J. **Grizzle WE**, Sexton KC, Bell WC. Human Tissue Biorepository. *In: Molecular Genetic Pathology*, volume 1, 2nd edition. (Eds. L Cheng, D. Zhang, Eble J), Springer Science+Business Media. 2013;483-497.
K. Bledsoe MJ, **Grizzle WE**. Use of Human Tissue in Research: The Current US Regulatory, Policy, and Scientific Landscape. Diagnostic Path 2013 (in press).
L. Zhang HG, **Grizzle WE**. Exosomes: a novel pathway of local and distant intercellular communication that facilitates the growth and metastasis of neoplastic lesions. Am J Pathol (in press)
M. Theodore SC, Davis M, Zhou F, Wang H, Rhim J, Turner, Ji W, Zeng G, **Grizzle WE**, Yates C. Genome-wide microRNA profiling of novel African American and Caucasian prostate cancer cell lines reveals that miR-152 is silenced by DNA methylation. Mol Cancer (submitted).
N. **Gaston SM**, Hayek JE, Otto G, Yen J, Bigley J, Neste LV, Kearney GP. Epigenetic field effect markers are indicative of occult high grade prostate cancer. Presented at the 2013 ASCO Genitourinary Cancers Symposium.
O. **Grizzle WE**. It is primarily the control of transcription and post-transcriptional processing that are critical to the development and progression of sporadic neoplasias. Biotech Histochem 2013; 88(7):361-4.

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Post-transcriptional processing of genetic information and its relation to cancer

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Abstract

During the development, progression and dissemination of neoplastic lesions, cancer cells hijack normal pathways and mechanisms, especially those involved in repair and embryologic development. These pathways include those involved in intercellular communication, control of transcription, post-transcriptional regulation of protein production including translation of mRNAs, post-translational protein modifications, e.g., acetylation of proteins, and protein degradation. Small, non-translatable RNAs, especially microRNAs (miRs), are Important components of post-transcriptional control. MiRs are produced from areas of the genome that are not translated into proteins, but may be co-regulated with their associated genes. MiRs bind to the 3′ untranslated regions of mRNAs and regulate the expression of genes in most cases by either promoting the degradation of mRNA and/or inhibiting the translation of mRNAs into proteins; thus, miRs usually cause a decrease in protein levels that would be expected if the mRNAs were translated normally. It is early in our understanding of how miRs affect neoplastic processes, but miRs are expressed differentially in most cancers and have been associated with tumor progression, chemoresistance and metastasis. MiRs are present in nanovesicles, such as exosomes, and thus are likely involved in intercellular communication, especially in neoplasia. MiRs are attractive targets for novel therapies of cancer as well as potential biomarkers that might be useful for early detection and diagnosis, and for prediction of therapeutic efficacy. MiRs also could aid and in determining prognosis, evaluating novel therapies, and developing preventive strategies by their use as surrogate end points.

Key words: biomarkers, mRNA, microRNA, post-transcriptional regulation, transcriptional regulation, translation

Transcription is the process by which sequences of the genome are read by the enzyme RNA polymerase II and pre-forms of messenger ribonucleic acid (mRNA) are produced. These pre-forms of mRNA (pre-mRNA) are converted into mRNA and the mRNAs subsequently are translated into proteins by ribosomes that match the sequences of the mRNA with triplet codes on transfer RNAs (tRNAs), each of which carries a unique amino acid. The complex of ribosomes, mRNA and tRNAs translate mRNA into amino acids that are bound together in a growing polypeptide chain ultimately to become a protein that matches the mRNA. Based on studying the genomics of prokaryotes, the concept arose that "one gene" was translated into "one protein"; however, based on the study of eukaryotic genomics, it is recognized that one gene ultimately can generate many different proteins. Therefore, fewer than 40,000 different genes may produce hundreds of thousands of unique proteins. In general, proteins generate the phenotypes of normal and diseased cells and tissues.

Because each cell type has specific roles and functions at multiple levels, i.e., organism, tissue and cell, the abundance, variety and type of

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proteins in each cell type may vary and may be cell-specific. Thus, a hepatocyte of the liver must be able to run the metabolic machinery necessary to produce glucose by gluconeogenesis and also produce and secrete many different proteins, e.g., albumin. In addition, the liver converts some waste products into bile. By contrast, skeletal muscle cells use glucose to power contractile proteins so that these cells can change size and shape rapidly. The cells of skeletal muscle do not produce glucose, albumin or bile. Because the DNA code is the same in each cell of an organism, accurate maintenance of the selective phenotypic characteristics in different types of cells is accomplished by genetic and epigenetic control of transcription, post-transcriptional processing including control of translation, and post-translational modifications of proteins.

In general, the mRNA produced from DNA in prokaryotic cells reproduces the base pattern of the complementary strand of the DNA. In eukaryotic cells, the DNA of a gene is separated into transcribed exons and untranscribed introns, i.e., the codes of introns are not incorporated into the final mRNA. Introns and other untranscribed areas of DNA may control the transcription of the gene or produce small RNAs that may regulate mRNA. In eukaryotic cells, a precursor form of mRNA (pre-RNA), which includes the RNA codes of introns and exons, initially is transcribed from the DNA, then the pre-mRNA is edited to remove the regions of the mRNA that correspond to the introns. During processing of pre-mRNA, specific exons may or may not be included in the final mRNAs, which results in "splice variants" of proteins. Similarly, recent studies have shown that there may be additional editing of mRNAs by unknown mechanisms so that some mRNAs may not mirror the DNAs from which they were originally transcribed (Li et al. 2011); however, these results are controversial.

Post-transcriptional regulation

After transcription, many processes (Table 1) may occur to produce the large number of proteins that make up both the cellular and extracellular components of a tissue, hence its phenotype. These processes produce many changes in the proteins that would not be predicted based on the code and/or structure of the DNA. Of the post-transcriptional processes, we focus here on how small RNAs regulate mRNA, therefore the amount of proteins produced from specific genes.

MicroRNAs (miRs)

MiRs are short (hence the name), approximately 22 nucleotide forms of RNA that are single-stranded; they are not translated into proteins. By affecting the degradation and translation of mRNA, however, miRs can modulate levels of proteins.

For convenience, microRNA usually is abbreviated miR. We will use miR for this class of molecules and mir as a prefix to denote specific human precursor forms of miRs (pre-miRs), which ultimately may be processed into the same mature miRs; thus an example of a mature miR is miR-let7 while an immature form would be mir-let7. To date, miRs have been described in most cells except some types of plants, e.g., certain marine plants and some fungi (Lee et al. 2010). The functions of miRs may vary in plants and lower organisms; however, we will focus primarily on the importance of miRs in mammals.

MiRs were discovered in the worm, *C. elegans*. In this worm, the extent of the expression of the heterochronic gene, lin-14, that regulates developmental timing critical for larval transition (Chalfie et al. 1981) occurs by the complementary binding of a small RNA, lin-4, to the 3′ untranslated region

Table 1. Post-transcriptional regulation in eukaryotes

Editing of the pre-mRNA
Alternate splicing of the pre-mRNA to include or exclude specific exons
Editing of the mRNA by enzymes, e.g., adenosine, to inosine
Editing of the mRNA by undefined mechanisms, changing the code of the mRNA and ultimately the structure of the proteins expected based on the DNA
Modulation of movement of mRNA out of the nucleus
Modification of the mRNA by small forms of RNA, including miRs
Degradation of the mRNA including control of degradation by miRs
Incorporation of atypical coded bases in tRNA, e.g., inosine, and their exit from the nucleus
Control of translation of mRNA to protein by miRs and by ribosomes, e.g., protein interactions with the internal ribosomal entry site (IRES)
Primary post-translational modifications of the initial proteins
Control of proteins by degradation

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(3′-UTR) of the lin-14 mRNA (Lee et al. 1993, Wightman et al. 1993). Subsequently, a small non-coding RNA, let-7, in *C. elegans,* was identified as a critical regulator of cellular development, which suggests that these miRs may act as fundamental developmental regulators (Reinhart et al. 2000). Ultimately, RNA molecules of the let-7 type were found to be conserved in many species including humans. With this observation, it became apparent that regulatory small RNAs were a general biological mechanism for post-transcriptional control of genetic information. MiRs function in the normal development and growth of cells from plants to man. Most miRs target developmental processes that involve cellular control, proliferation, and cell death. By contrast, processes that characteristically involve routine maintenance functions common to all cells typically are not controlled by miRs. At present, it is believed that about 1/3 of the human genome may be under transcriptional regulation by miRs (Chen 2005, Phillips 2008, McDaneld 2009).

Production of miRs

Typically, the genes that produce miRs can be located in an anti-sense orientation to exons or introns or in areas of DNA that were thought to be non-coding. Nevertheless, the genes that produce miRs are regulated by promoters and other regulatory mechanisms. Also, some genes for miRs may be in a sense orientation within introns or other non-coding areas of the DNA. This orientation within genes permits a miR to be co-regulated with its "related gene." For example, a genetic sequence that codes for a specific miR, e.g., microRNA-xxy, which regulates the mRNA transcribed from gene y is located within the intron between exons 4 and 5 of the gene for y. Thus, as gene y is transcribed, the miR that binds to the mRNA of gene y would be transcribed with y and would be regulated, in part, by factors that control the transcription of y.

MiRs usually are produced from specific genes by RNA polymerase II (less commonly by RNA polymerase III) as a "primary miR" (pri-miR) that contains hundreds of nucleotides and a poly A tail at the 3′ end. When pri-miR is from a transcribed gene, pri-miR may be separated in the course of splicing, e.g., removing the intron areas of the mRNA. RNase III enzymes, Drosha and Pasha, enzymatically generate from the pri-miR approximately a 70 base form of RNA designated pre-miR, which leaves the nucleus as a complex with Exportin-5 (Chen 2005, Phillips 2008, McDaneld 2009). The structure of the pri- and pre- forms of miR, though short, includes a hairpin loop. Once processed to miR, the hairpin loop is removed. The pre-miR then is cleaved by an RNA III enzyme, dicer, and the cleaved form of RNA is incorporated into an Argonaute-protein-containing complex called an RNA induced silencing complex or RISC. When bound as part of the RISC, the RNA is composed of two complementary strands. One strand then is cleaved, released by the RISC and degraded. The RISC orients the remaining strand (now designated a mature miR) so that it can bind optimally to target areas of mRNAs (Chen 2005, Phillips 2008, McDaneld 2009).

Functions of miRs

The target areas of miRs in most cases are specific sequences of the 3′-UTR of the mRNA. The same RISC-miR complex can bind and regulate many different mRNAs if they have the same or similar sequences in their 3′-UTRs so that the same miR can modulate concomitantly many different mRNAs (e.g., 100) (Chen 2005, Phillips 2008, McDaneld 2009).

If there is a strong complementary Watson-Crick match with the bases of the target region of a mRNA, the mRNA is cleaved by an energy requiring (ATP) process so that the poly A end of the 3′ mRNA and the capped 5′ end of the RNA are removed, which enables rapid mRNA degradation of each fragment of the mRNA by exonucleases (Chen 2005, Phillips 2008, McDaneld 2009). The RISC and its miR are stable and continue to be biologically active so it can bind other mRNA molecules (Chen 2005, Phillips 2008, McDaneld 2009). Unlike mRNAs, mature miRs are thought to be very stable in vitro as well as in vivo; in addition, miRs can be identified in fixed and paraffin embedded tissues so archival paraffin blocks are used for their analysis (Bovell et al. 2012).

If the miR does not have a strong base pairing with a sequence of the 3′-UTR, it still may bind, but less avidly, to the target mRNA. In such cases, the binding may not result in cleavage of the mRNA, but the bound miR inhibits the translation of the mRNA and sets up the mRNA for eventual degradation by the transfer of the mRNA to processing bodies or "P-bodies," the sites where most mRNAs, whether regulated by miRs or not, are destroyed or are stored prior to degradation (Chen 2005, Phillips 2008, McDaneld 2009). MiRs also may act in other ways to affect genetic information. For example, they may bind to regulatory introns to modulate transcription and/or miRs may inhibit

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translation of mRNAs (Chen 2005, Phillips 2008, McDaneld 2009).

It is important to understand how miRs are identified to interpret the literature. As indicated, "mir" precedes the name of a pre-miR while "miR" precedes the designation of a mature miR. Over the years, specific miRs have been numbered to distinguish and identify them. Usually, the numbers range from 1 to 9999; the small numbers designate miRs that were identified earlier. An initial three letter prefix may refer to species associated with the miR. Some species designations are listed in Table 2. Thus the designation for miR 130 in humans could be "hsa-miR-130," while the miR 130 in mice would be designated "mmu-miR-130." A pre-miR may produce miRs from different ends of the molecule; if one of two miRs comes from the 3′ end, it is designated, -3p, and if from the 5′ end, -5p.

When mRs differ by only one or two nucleotides from the form of miR identified originally, the related miRs are designated by a letter, e.g., "a," "b," "e," and in some cases where there are three very similar miRs as "b#." Also, if two miRs come from the same pre-miR, but one is the minor component, it may have been labeled previously as "*." Thus, miR-130* is the minor component and miR-130 is the major form of miR-130 in a cell; however, designations recently have been changed to -3p or -5p to describe such forms. Examples of various designations of miRs are shown in Fig. 1.

Frequently the species designation, hsa, is omitted for miRs for studies of human specimens and species prefixes often are deleted if a publication is limited to one species. Of great use to investigators studying miR is the web site, "mirbase.org," currently 2011 version 18, that can be used to search for specific miRs or information about miRs. This web site contains more than 18,000 entries concerning more than 150 species and is organized to be accessed using several approaches. One approach is based on "species: chromosome: sequences." If one enters *Equus caballus* (horse), for example, and chromosome 2, 18 "mirs" are listed, and each represents the pre-form of the miR. Specifically, one finds eca-mir-30e on chromosome 2. The mirbase.org site was less useful, however, for searching for miRs involved in cancer. By contrast, the "Human MicroRNA Disease Database" and the "miR2 disease database" are much more useful concerning the literature related to the involvement of miRs in diseases in general and neoplasia specifically (Jiang et al. 2008, Lu et al. 2008). These databases can be searched by organ, cancer or type of cancer, e.g., carcinoma, but sometimes must be searched under "neoplasia."

Table 2. Species and designation for miR

Species	Designation
human	hsa
mouse	mmu
rat	rno
sheep	oar
dog	cfa
chicken	gga
viral	__v
Drosophilia	d__

Frequently, miRs provide a mechanism by which the amounts of proteins can be down-regulated. This function of miRs may occur by facilitating the degradation of mRNAs, which inhibits the translation of mRNAs or the transcription of mRNAs. Sometimes the mRNAs and their associated proteins that are down-regulated are involved primarily in the metabolism or degradation of important driver genes. For example, some proteins are controlled primarily by the degradation of the protein product (e.g., $p27^{kip\text{-}1}$ metabolized by Skp-2); thus, if miRs inhibit the metabolic enzyme targeting a phenotypically important protein, the phenotypically important molecule would be expected to increase. Similarly, miRs can be inhibited by methylation of their promoters as well as by proteins from other genes, and less commonly, may stimulate translation directly and thus increase specific proteins (Vasudevan et al. 2007).

Because miRs function in the normal development and growth of cells, they would be expected to be dysregulated in disease processes and are likely to be important for at least some aspects of all human diseases. We are just beginning to understand their importance in human diseases (Jiang et al. 2008, Lu et al. 2008). As expected, because miRs frequently are involved in developmental processes, congenital malformations of many organ systems, including the heart and brain, may occur if miRs are dysregulated.

Abnormalities related to miRs may occur at several levels including deregulation of the pri-miR, pre-miR, or mature miR. Other types of dysregulation may occur by modifications of the target 3′-UTR of mRNAs that affect the binding of miRs. Specific changes in the mRNA may cause the 3′-UTR no longer to bind a specific miR or it may cause binding to the 3′-UTR of a previously unbound miR. All the possibilities above may occur as somatic mutations or gene dysregulation during the development and progression of specific cancers or as germ line mutations by which such abnormalities are transmitted to offspring and result in familial diseases.

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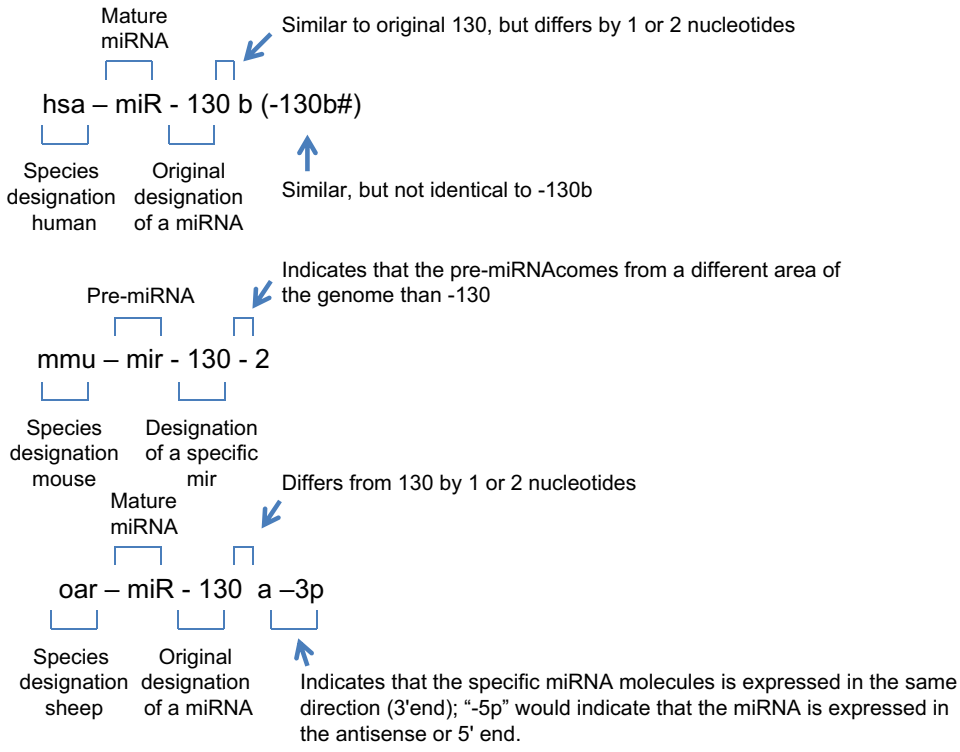

Fig. 1. The above cartoon demonstrates some of the conventions used in labelling various forms of microRNAs. These conventions have changed over the years so care should be taken to ensure the specific identities of the microRNAs being studied.

MiRs and exosomes

MiRs represent one of several newly described categories of molecules and pathways that "fine tune" cellular functions. MiRs can be transcribed and function not only within cells, but they also can be transferred to cells as a form of intercellular communication. This can be accomplished by packaging of miRs in membrane bound vesicles that are released from cells into the interstitial space. The vesicles within interstitial spaces may act locally through autocrine, paracrine or other-crine activities, or they may be picked up by blood or other bodily fluids to provide endocrine-like signals to distant cells (Kosaka and Ochiya 2012). The exosome is one type of vesicle that has been reported to contain molecules of miR. Exosomes are bilayered-membrane-bound nanovesicles that are released from the vesicular bodies of normal and diseased cells. Exosomes are present in most body fluid including blood. In blood, exosomes typically are 30–100 nm in diameter, have been described as "cup-shaped" and they express specific molecules such as the tetraspanins, CD9, CD63 and CD81. Although the details are uncertain, the molecular features of the external surfaces of exosomes control their uptake by cells, hence the effects of their contents on cellular functions (Zhang and Grizzle 2011).

Exosomes have been reported to contain functional proteins, mRNAs and lipids as well as miRs. Again, the details about how these molecules are sorted selectively into exosomes remains elusive. Nevertheless, typical exosomes contain hundreds of miRs and the packaging of miRs in exosomes may protect them from RNases (Kosaka and Ochiya 2012). How specific miRs in exosomes function and provide signals to cells is uncertain.

The molecules contained in exosomes, especially the proteins, peptides and mRNA, have been reported to be characteristic of the cells from which they arise. Because tumors secrete exosomes, i.e., tumor-derived (TD) exosomes, their contents, especially proteins, peptides, mRNA and miRs, have been found to be characteristic of the tumors from which they arise. Because TD exosomes contain miRs, it has been proposed that these miRs can be used as biomarkers that are useful for translational research focused on diagnosis, risk assessment, prediction, measurement of therapeutic responses and determination of prognosis (Lu et al. 2005, Grizzle et al. 2012, Zhang and Grizzle 2013). Circulating TD-exosomes of patients with ovarian cancer

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have been reported to contain eight miRs whose expressions were significantly distinct from those observed in benign diseases (Taylor and Gercel-Taylor 2008). In many cases, the miRs were expressed differentially in exosomes compared to the matching fluids; thus, miRs contained within exosomes may provide greater sensitivity and specificity for translational studies (Kosaka and Ochiya 2012, Nair et al. 2012).

Other small RNAs

SiRNAs are double-stranded small RNAs that are present in prokaryotes, plants and animals evolutionarily below worms where they protect against certain viruses and intracellular parasites. In some cases, this double-stranded RNA attracts a protein complex containing dicer, which cleaves the double-stranded RNA. Like miRs, they are bound by Argonaute and other molecules into an RNA-induced silencing complex (RISC), which then is processed as described previously for miR and whose action may be similar to miRs. Alternatively, siRNAs may be bound with Argonaute into a different complex, the RNA induced transcriptional silencing complex (RITSC), which can inhibit transcription of genes.

There are other endogenous forms of small RNAs that also act post-transcriptionally on mRNA and are related to, but currently are considered distinct from, miRs (Lee et al. 2010, Naqvi et al. 2009). Small RNAs that mimic siRNA may be synthesized exogenously and used experimentally to decrease specific mRNAs, hence, proteins; these also typically are called siRNAs (Devi 2006).

MicroRNAs in cancer

Pre-invasive and invasive neoplastic cells typically hijack embryological and repair processes and pathways to facilitate neoplastic development and progression. Pathways controlling proliferation, apoptosis, cellular motility, cellular invasiveness and cellular orientation (polarity) are typical pathways that are dysregulated in neoplastic cells to facilitate their growth and survival. While pathways controlling proliferation and apoptosis, for example, are regulated carefully in normal tissues, these pathways frequently are dysregulated in neoplasia. Also, with increased proliferation and dysregulated apoptosis, mutations in genes, overexpression of genes, and changes in the epigenetic control of transcription may develop in neoplasia.

MiRs are involved in most aspects of neoplasia from the development of neoplastic lesions to the spread of cancer by metastasis. MiRs involved in cancer have been designated as "oncomiRs" (Cho et al. 2007, Esquila-Kerscher and Slack 2007, Lujambio 2009). OncomiRs can modulate the development, progression and dissemination of neoplastic processes by acting as either tumor suppressors (e.g., miR-34a) or oncogenes (e.g., mir-17-92 cluster). It is of interest that the tumor suppressors, miR-18a, miR-34b/c and miR-9, can be silenced by hypermethylation and this silencing facilitates nodal metastasis (Cho et al. 2007, Lujambio 2009).

MetastamiRs are miRs that are involved specifically in the metastatic process (Hurst et al. 2009, White et al. 2011, Lopez-Camarillo et al. 2012). They frequently function as an intermediate signal in pathways that inhibit or facilitate cancer, e.g., miR146a/b acts downstream of the BRMS1 gene, which suppresses metastasis in breast cancer, but miR146a/b acts prior to genes that are identified as regulated by BRMS1 (Hurst et al. 2009). Other miRs involved in metastasis of breast cancers include miR-335, miR-126, miR-10b, miR-9 and miR-155 (Tavazoie et al. 2007, Ma et al. 2007, 2010, Xiang et al. 2011). Similarly, miR-31 usually acts as a general tumor suppressor as well as a suppressor of metastasis by its action on integrin-α5, radixin and RhoA, while the miR-200 family (miR -200a, b, and c, miR-141 and miR-429) may inhibit or facilitate metastasis depending on whether effects of epithelial to mesenchymal transition or mesenchymal to epithelial transition dominate (Dykxhoom 2010).

MiRs have been claimed to be useful for early detection, diagnosis and prognosis of various cancers and for management of patients by prediction of therapeutic efficacy, monitoring responses to therapy or as targets for therapy (Mak et al. 2005, Waldman and Terzic 2007, Martello et al. 2010, Manne et al. 2010, Shah et al. 2009). It has been suggested that MiRs potentially are important clinically for most cancers and specifically for cancers of the ovary (Shah et al. 2009, Taylor and Gercel-Taylor 2008), lung (Rabinowitz et al. 2009, Mallick et al. 2010), breast (Pigati et al. 2010, Xiang X et al. 2011), pancreas (Wang et al. 2009, Srivastava et al. 2011), prostate (Coppola et al. 2010) and brain (Delfino et al. 2011).

Small non-translatable RNAs such as miRs now are recognized as an important group of regulatory molecules that are involved primarily in post-transcriptional regulation of genetic information.

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There are thousands of different miRs that bind to the untranslated 3′ ends of mRNAs and thereby modulate the degradation of these mRNAs and inhibit their translation. MiRs can be carried within exosomes to provide autocrine, paracrine, and endocrine type intercellular signals among normal and diseased cells, and especially neoplastic cells. MiRs also are involved in disease by their dysregulation. MiRs likely represent one of the biological pathways that neoplastic lesions use to promote their development, progression and dissemination; therefore, these molecules may be attractive targets for novel approaches to therapy, diagnosis and prevention of cancer.

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Preservation of immunorecognition by transferring cells from 10% neutral buffered formalin to 70% ethanol

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Abstract

Prolonged fixation of cells and tissues in 10% neutral buffered formalin (NBF) may decrease immunorecognition in some antigen-antibody pairs. Short fixation in 10% NBF followed by transfer to 70% ethanol has been used to overcome these effects, but the effects of this transfer on immunorecognition have not been explored adequately. We used two cell lines, DU145 (prostate cancer) and SKOV3 (ovarian cancer), grew them on coverslips and fixed them with 10% NBF at room temperature for 5 min and 12, 15, 18, 36, 108 and 180 h. Aliquots of the same cells were fixed in 10% NBF for 12 h, then transferred to 70% ethanol for 3, 6, 24, 96 and 168 h. Immunostaining with PCNA, Ki67-MIB-1, cytokeratins AE1/AE3 and EGFr was done concomitantly. In both cell lines, immunorecognition decreased between 18 and 36 h of fixation in 10% NBF for PCNA, Ki67-MIB-1 and cytokeratins AE1/AE3. By 108 to 180 h of 10% NBF exposure, there was complete loss of immunorecognition of PCNA and extensive loss of Ki67-MIB-1 and cytokeratins AE1/AE3. The effects on EGFr immunorecognition were less. Transfer to 70% ethanol after fixation for 12 h in 10% NBF preserved immunorecognition of the antibodies.

Key words: cytokeratins AE1/AE3, EGFr, 70% ethanol, 10% formalin, immunohistochemistry, Ki67-MIB-1, PCNA

Ten percent neutral buffered formalin (NBF) is used almost universally for diagnostic pathology because of its ability to preserve consistently the morphological details of cells and tissues (Grizzle et al. 2008, Grizzle 2009).

The reaction of 10% NBF with proteins in cells and tissues is thought to occur by three reactions. In the first reaction, formaldehyde reacts rapidly primarily with the amino and thiol groups of amino acids to form hydroxymethyl derivatives (Pearse 1968). Subsequently, in the case of primary amino groups, some hydroxymethyl groups may undergo a condensation reaction to form an imine, also called a Schiff base (Metz et al. 2004). This second reaction occurs more slowly. These two initial reactions may influence immunostaining based on the current approach to fixation, which typically limits fixation to less than 24 h for a thin (e.g., 3 mm) piece of tissue. As the time of exposure to 10% NBF increases, a third reaction occurs in which the imine reacts with other side chains including glutamine, asparagine, tryptophan, histidine, arginine, cysteine and tyrosine in a cross-linking fashion (Metz et al. 2004). These molecular changes may mask some antigen epitopes, which would reduce immunostaining (Arnold et al. 1996). The relatively slow penetration of formaldehyde (<1 mm/h) affects the fixation reactions in tissues. In addition, processing tissues fixed in 10% NBF to paraffin also affects immunorecognition (Grizzle et al. 2008, Otali et al. 2009).

Fixation by 10% NBF for longer than 48 h has been studied extensively, but less is known concerning fixation in 10% NBF for shorter periods, e.g., <12 h. Also, the effects of various concentrations of

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Table 1. Duration of fixation in 10% NBF compared to fixation for 12 h in 10% NBF followed by transfer to 70% ethanol

Fixation in 10% NBF (h)	Fixation in 10% NBF for 12 h and transfer to 70% ethanol (h)
0.083	–
12	–
15	12 + 3
18	12 + 6
36	12 + 24
108	12 + 96
180	12 + 168

ethanol following initial fixation in 10% NBF, has not been studied adequately (Otali et al. 2009). It has been reported anecdotally and generally accepted that transferring thin tissues and cells that have been fixed in 10% NBF for more than 24 h to 70% ethanol prevents further loss of immunorecognition of some epitopes. There are few studies in the literature, however, to support this belief and to our knowledge, there is no study of the optimal time for transferring tissues from 10% NBF to 70% ethanol (Leung et al. 2011).

Ethanol is a dehydrating agent, but it also may act on the hydroxymethyl adducts that have not been cross-linked to catalyze the formation of reactive imines by removing the hydrogen atom from the nitrogen of the original amine end group and the hydroxyl group from the 10% NBF adduct (Dapson 2007).

Immunorecognition by monoclonal and polyclonal antibodies likely depends on many factors including how both the epitopes/antigens and the antibody identifying the epitopes react; this is indicated by the differential effects of fixation in 10% NBF and/or other cross-linking fixatives that may modify the epitope (Arnold et al. 1996). Other less commonly considered effects of cross-linking fixatives that may affect immunorecognition include steric hindrance of epitope-antibody reactions resulting from cross-linking and local chemical effects caused by reactive hydroxymethyl groups and/or imines.

Previously, we reported that fixation interacts with tissue processing to decrease immunorecognition (Otali et al. 2009). In that study, cells grown on microscope slides were processed to paraffin as a model to study the interaction of fixation by 10% NBF with cumulative processing steps to paraffin. In the study reported here, cells were grown on coverslips, because this approach frequently is used in research and constitutes a simplified model for enhancing our understanding of the potential short term effects of 10% NBF fixation and transfer to 70% ethanol.

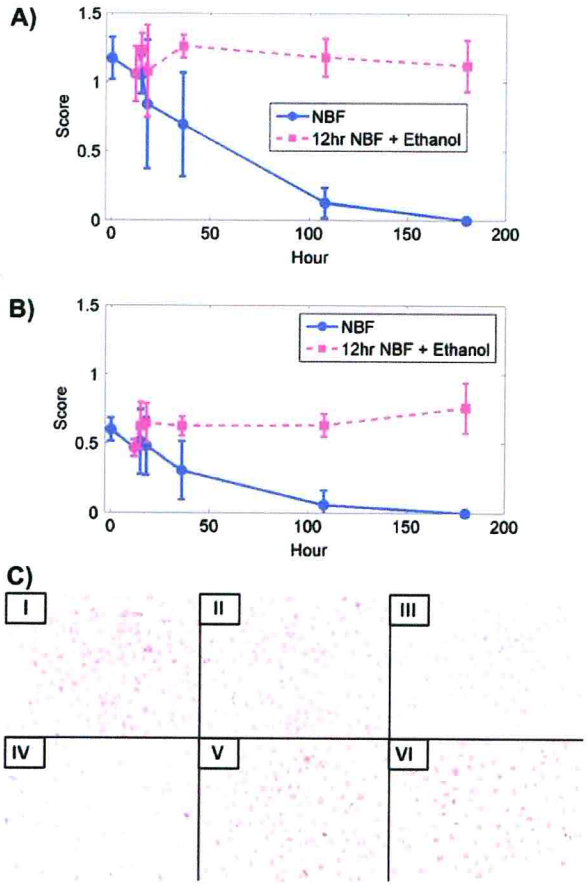

Fig. 1. Immunostaining scores for PCNA. A) PCNA staining in DU145 cells after fixation in 10% NBF at times matched with fixation in 10% NBF for 12 h followed by transfer to 70% ethanol at comparable total exposure times. B) PCNA staining for SKOV3 cells after fixation in 10% NBF at times matched with fixation for 12 h in 10% NBF followed by transfer to 70% ethanol for comparable total times of exposure. Data from both cell lines are from three replicate experiments. Error bars are standard deviations. C) DU145 cells after staining for PCNA. × 200. I, II, III, IV, V and VI represent 5 min, 12 h, 36 h, 180 h 10% NBF, and 12 h in 10% NBF + 24 h in 70% ethanol, and 12 h in 10% NBF + 168 h 70% ethanol, respectively.

We grew cells on coverslips specifically to evaluate whether transfer of cells from 10% NBF to 70% ethanol decreases the loss of immunorecognition caused by fixation in 10% NBF. Our study also was designed to determine the optimal time for transfer of cells from 10% NBF to 70% ethanol and how various times in ethanol affect immunorecognition. The results suggest that there is a clear benefit to transferring cells, and potentially tissues, from 10% NBF to 70% ethanol prior to immunohistochemical analysis.

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Table 2. *P*-values of t-tests between any sample pairs for immunostaining score for PCNA in DU145 and SKOV3 cells fixed in 10% NBF

	Time (h) in 10% NBF	0.083	12	15	18	36	108	180
DU145	0.083	–	0.05	0.02	<0.01	<0.01	<0.01	<0.01
	12	–	–	0.46	0.05	<0.01	<0.01	<0.01
	15	–	–	–	0.05	<0.01	<0.01	<0.01
	18	–	–	–	–	0.18	<0.01	<0.01
	36	–	–	–	–	–	<0.01	<0.01
	108	–	–	–	–	–	–	<0.01
SKOV3	0.083	–	<0.01	0.09	0.02	<0.01	<0.01	<0.01
	12	–	–	0.77	0.59	<0.01	<0.01	<0.01
	15	–	–	–	0.34	<0.01	<0.01	<0.01
	18	–	–	–	–	0.02	<0.01	<0.01
	36	–	–	–	–	–	<0.01	<0.01
	108	–	–	–	–	–	–	0.02

Data are from three replicate experiments.

Material and methods

The effects of fixation of cells by 10% NBF for 5 min, 12, 15, 18, 36, 108 and 180 h were compared with fixation for 12 h in 10% NBF followed by transferring the cells to 70% ethanol for 3, 6, 24, 96 and 168 h so that the total time in the two solutions would be equivalent (Table 1). Fixation for 5 min was considered minimal, because "no fixation" usually resulted in detachment of cells from the coverslips during immunostaining.

Two cell lines were used: DU145 (prostate cancer) and SKOV3 (ovarian cancer) obtained from American Type Culture Collection (ATCC). The cell lines were maintained in RPMI 1640 and DMEM, respectively, with 10% fetal calf serum plus supplements, MEM vitamin solution (Gibco, Grand Island, NY) l-glutamine (Gibco), antibiotic-antimycotic solution of penicillin, streptomycin and amphotericin B (Gibco) in an incubator with 5% CO_2 at 37° C.

DU145 or SKOV3 cell lines were trypsinized, re-suspended in their respective media and plated uniformly on 22 × 22 mm sterile coverslips in six-well plates at a cell concentration of 150,000/ml for DU145 and 175,000/ml for SKOV3. Plating was carried out over several days in a decreasing time schedule. When confluence reached about 70%, usually after two days, the cells on the

Table 3. *P*-values of t-tests for immunostaining score between 12 h of 10% NBF fixation or 10% NBF for 12 h followed by transfer to 70% ethanol at experimental time points for DU145 and SKOV3 after cells

	12 (h) in 10% NBF + EtOH (h)	12 + 0	12 + 3	12 + 6	12 + 24	12 + 96	12 + 168
PCNA							
DU145	12 + 0	–	1.0	0.58	1.0	0.97	0.80
SKOV3	12 + 0	–	1.0	1.0	1.0	1.0	1.0
Cytokeratins AE1/AE3							
DU145	12 + 0	–	0.20	0.18	0.72	0.07	0.25
SKOV3	12 + 0	–	0.12	0.52	0.77	0.86	0.37
EGFr cytoplasmic							
DU145	12 + 0	–	0.96	0.99	0.98	0.99	0.81
SKOV3	12 + 0	–	1.0	0.99	1.0	0.98	1.0
EGFr membrane							
DU145	12 + 0	–	1.0	1.0	1.0	1.0	1.0
SKOV3	12 + 0	–	1.0	1.0	1.0	1.0	1.0
Ki67-MIB-1							
DU145	12 + 0	–	0.36	1.0	0.99	0.98	0.82
SKOV3	12 + 0	–	1.0	1.0	1.0	1.0	1.0

Data are from three replicate experiments.

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Table 4. *P*-values of t-tests for percentage of DU145 and SKOV3 cells staining for PCNA, cytokeratins AE1/AE3 and EGFr; values are comparisons between 10% NBF fixation for 5 min and each subsequent experimental time point

	Time in 10% NBF (h)	0.083	12	15	18	36	108	180
PCNA								
DU145	0.083	–	0.94	0.92	0.50	0.10	<0.01	<0.01
SKOV3	0.083	–	0.96	0.86	0.65	0.03	<0.01	<0.01
Cytokeratins AE1/AE3								
DU145	0.083	–	0.72	0.33	<0.01	<0.01	<0.01	<0.01
SKOV3	0.083	–	0.80	0.66	0.97	<0.01	<0.01	<0.01
EGFr cytoplasmic								
DU145	0.083	–	0.57	0.43	0.64	0.56	0.08	<0.01
SKOV3	0.083	–	0.85	0.73	0.50	1.0	0.97	0.98
EGFr membrane								
DU145	0.083	–	0.04	0.32	<0.01	<0.01	<0.01	0.01
SKOV3	0.083	–	0.31	0.02	0.02	0.08	<0.01	0.01

Data are from three replicate experiments.

Table 5. *P*-values of t-tests for percentage of DU145 and SKOV3 cells staining; values are comparison between 10% NBF fixation for 12 h with 10% NBF fixation plus subsequent experimental time points in 70% ethanol

	Time in 10% NBF (h) + EtOH (h)	12 + 0	12 + 3	12 + 6	12 + 24	12 + 96	12 + 168
PCNA							
DU145	12 + 0	–	0.98	0.79	0.68	1.0	0.89
SKOV3	12 + 0	–	0.89	1.0	1.0	1.0	1.0
Cytokeratins AE1/AE3							
DU145	12 + 0	–	0.01	0.22	0.88	0.53	0.88
SKOV3	12 + 0	–	0.16	0.35	0.95	0.98	1.0
EGFr cytoplasmic							
DU145	12 + 0	–	1.0	1.0	0.84	0.94	1.0
SKOV3	12 + 0	–	1.0	0.99	1.0	0.98	1.0
EGFr membrane	12 + 0	–	0.63	0.99	0.98	1.0	0.92
	12 + 0	–	0.94	0.91	1.0	0.99	1.0

Data are from three replicate experiments.

Table 6. *P*-values of t-tests between any sample pairs for immunostaining score for cytokeratins AE1/AE3 in DU145 and SKOV3 cells fixed in 10% NBF

	Time (h) in 10% NBF	0.083	12	15	18	36	108	180
DU145	0.083	–	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
	12	–	–	0.55	<0.01	<0.01	<0.01	<0.01
	15	–	–	–	<0.01	<0.01	<0.01	<0.01
	18	–	–	–	–	<0.01	<0.01	<0.01
	36	–	–	–	–	–	<0.01	<0.01
	108	–	–	–	–	–	–	<0.01
SKOV3	0.083	–	0.15	<0.01	<0.01	<0.01	<0.01	<0.01
	12	–	–	<0.01	0.05	<0.01	<0.01	<0.01
	15	–	–	–	0.93	0.10	<0.01	<0.01
	18	–	–	–	–	<0.01	<0.01	<0.01
	36	–	–	–	–	–	<0.01	0.02
	108	–	–	–	–	–	–	0.86

Data are from three replicate experiments.

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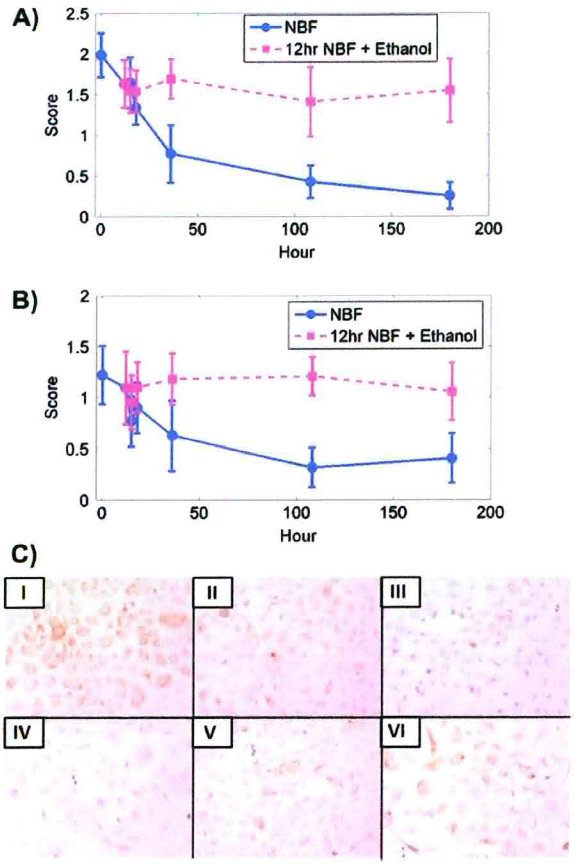

Fig. 2. Immunostaining scores for cytokeratins AE1/AE3. A) Immunostaining for cytokeratins AE1/AE3 in DU145 cells after fixation in 10% NBF at times matched with fixation in 10% NBF for 12 h followed by transfer to 70% ethanol for comparable total times of exposure. B) Immunostaining for cytokeratins AE1/AE3 in SKOV3 cells after fixation in 10% NBF at times matched with fixation in 10% NBF for 12 h followed by transfer to 70% ethanol for comparable total times of exposure. Data from both cell lines are from three replicate experiments. Error bars are standard deviations. C) Immunostaining of DU145 cells for cytokeratins AE1/AE3. I, II, III, IV, V and VI represent 5 min, 12 h, 36 h, 180 h in 10% NBF, and 12 h in 10% NBF + 24 h in 70% ethanol and 12 h in 10% NBF + 168 h in 70% ethanol, respectively. × 200.

coverslips were rinsed quickly twice in PBS, pH 7.4, and either fixed in 10% NBF alone (Richard Allan Scientific, Kalamazoo, MI) for 5 min, 12, 15, 18, 36, 108 or 180 h or fixed in 10% NBF for 12 h, then placed in 70% ethanol (AAPER Alcohol and Chemical Co. Shelbyville, KY) for 3, 6, 24, 96 or 168 h (Table 1). Fixation was carried out at room temperature and all fixation times were synchronized to enable immunostaining of all experimental variations to be performed at the same time.

When the designated end point of fixation had been reached, the fixed cells on the coverslips were rinsed in Tris buffer, pH 7.6, for 10 min and permeabilized. For the permeabilization step, the cells on the coverslips were dehydrated through graded concentrations of ethanol, i.e., 70, 95%, and absolute ethanol for 2 min at each concentration, treated with acetone (Fisher Scientific, Fairlawn, NJ) for 15 sec, then rehydrated through graded concentrations of ethanol, i.e., absolute, 95, and 70%, before washing in Tris buffer for 2 min (Rodriguez-Burford et al. 2002). Endogenous peroxidase was quenched by exposure to 3% aqueous H_2O_2 for 5 min and rinsing with Tris buffer. To reduce nonspecific staining, 3% goat serum was added to the cells on the coverslips for 1 h at room temperature. The cells on the coverslips were stained with a monoclonal antibody to the proliferative nuclear marker, PCNA (Santa Cruz Biotechnology, Santa Cruz, CA), diluted 1:18,000, a monoclonal antibody to the proliferative biomarker, Ki67, clone MIB-1 (Bio-genex, San Ramon, CA) diluted 1:60, monoclonal antibodies to anti-keratins AE1/AE3 (Boehinger Mannheim Corp., Indianapolis, IN) 5 µg/ml, diluted 1: 40 and a monoclonal antibody to EGFr (Zymed, San Francisco, CA) 3 µg/ml, diluted 1:5. Dilutions of antibodies were in phosphate buffer EDTA (PBE), pH 7.6. These relatively dilute concentrations of antibodies were chosen so that staining was relatively weak to make assessment of effects on immunorecognition easier. For each antibody and cell line, a control was included in which the primary antibody was replaced with 3% goat serum. Next, the cells on the coverslips were rinsed with Tris buffer, pH 7.6, and incubated with multispecies biotinylated goat anti-mouse/rabbit secondary antibody for 10 min (Signet, Dedham, MA) and HRP-conjugated streptavidin for 5 min (Signet). Color was developed with diaminobenzidine (DAB) for 7 min (Biogenex, San Ramon, CA) to produce an insoluble chromogen. The cells on the coverslips were rinsed with deionized water, counterstained with Mayer's hematoxylin (Sigma-Aldrich, St. Louis, MO) for 1 min 15 sec, blued in tap water, dehydrated though graded concentrations of ethanols: 70, 95%, and absolute ethanol, before clearing in thee changes in xylene (Fisher Scientific, Fairlawn, NJ). The cells on the coverslips were mounted on microscope slides using Permount (Fisher Scientific). Immunostaining for each experiment was repeated independently three times.

Blinded evaluations were performed by a board certified diagnostic pathologist (W.E.G.). The immunostaining for a specific cell line for all three experiments under each condition was evaluated during

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Table 7. *P*-values of t-tests between any sample pairs for cytoplasmic immunostaining score of EGFr in DU145 and SKOV3 cells fixed in 10% NBF

	Time (h) in 10% NBF	0.083	12	15	18	36	108	180
DU145	0.083	–	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
	12	–	–	0.37	0.09	0.03	<0.01	<0.01
	15	–	–	–	0.13	0.04	<0.01	<0.01
	18	–	–	–	–	0.39	<0.01	<0.01
	36	–	–	–	–	–	<0.01	<0.01
	108	–	–	–	–	–	–	<0.01
SKOV3	0.083	–	0.05	<0.01	<0.01	0.18	<0.01	0.02
	12	–	–	<0.01	0.01	0.80	0.08	0.26
	15	–	–	–	0.87	1.0	0.94	1.0
	18	–	–	–	–	1.0	0.75	0.95
	36	–	–	–	–	–	0.02	0.07
	108	–	–	–	–	–	–	0.79

Data are from three replicate experiments.

the same session. Two parameters were evaluated: percentage of cells stained and immunostaining score as described in (Grizzle et al. 1998, Otali et al. 2009). Briefly, the intensity of nuclear staining was determined in 1.0 increments from 0 for no staining to 4 for strongest staining. The proportion of cells stained at each intensity level was estimated and multiplied by the staining intensity. The total immunostaining score is the sum of the products of the proportion of cells stained at each staining intensity multiplied by the staining intensity, e.g., 40% of cells staining at intensity of 2 would yield $0.4 \times 2 = 0.8$ and 60% of cells staining at 3 would yield $0.6 \times 3 = 1.8$. Adding the components of the immunostaining score for this example would be 2.6. Similarly, 100% of cells with no staining would give a total immunostaining score of $1 \times 0 = 0$, and 100% of cells staining at maximum intensity would give an immunostaining score of $1 \times 4 = 4$, thus immunostaining scores range from 0 to 4. Five random fields were evaluated for each variable for each experiment and the values obtained from the three replicate independent experiments were used to calculate means and standard deviations. Where appropriate, intracellular localization was evaluated separately, e.g., EGFr cellular membrane staining was evaluated separately from cytoplasmic staining for EGFr. Because of the pattern of staining of Ki67-MIB-1 at low concentrations, only staining of mitotic cells was evaluated.

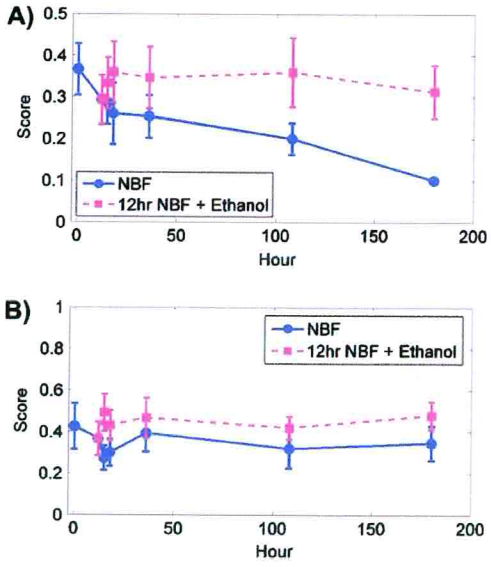

Fig. 3. Cytoplasmic immunostaining scores for EGFr. A) DU145 cells after fixation in 10% NBF at times matched with fixation in 10% NBF for 12 h followed by transfer to 70% ethanol at comparable total times of exposure. B) SKOV3 cells after fixation in 10% NBF at times matched with fixation for 12 h in 10% NBF followed by transfer to 70% ethanol at comparable total times of exposure. Data from both cell lines are from three replicate experiments. Error bars are standard deviations.

Because our hypothesis was based on prior studies that indicated that the percentage of cells stained and the immunostaining score would decrease with longer fixation in 10% NBF, right-tailed pair-wise t-tests were performed to assess the differences in immunostaining between experimental time periods. We considered the difference statistically significant if $p \leq 0.01$. As expected, we observed a general trend toward a decreasing immunostaining score with increasing duration of fixation in 10% NBF. In addition, preliminary data indicated that transfer of specimens into 70% ethanol would minimize the decreased

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Table 8. *P*-values of t-tests between any sample pairs of membrane immunostaining score for EGFr in DU145 and SKOV3 cells fixed in 10% NBF

	Time (h) in 10% NBF	0.083	12	15	18	36	108	180
DU145	0.083	–	0.04	0.02	<0.01	0.06	<0.01	<0.01
	12	–	–	0.31	0.09	0.37	<0.01	<0.01
	15	–	–	–	0.17	0.50	<0.01	<0.01
	18	–	–	–	–	0.77	0.08	<0.01
	36	–	–	–	–	–	0.03	<0.01
	108	–	–	–	–	–	–	<0.01
SKOV3	0.083	–	0.86	0.78	0.61	0.93	0.09	0.27
	12	–	–	0.42	0.19	0.66	0.02	0.06
	15	–	–	–	0.29	0.72	0.04	0.11
	18	–	–	–	–	0.90	0.05	0.19
	36	–	–	–	–	–	<0.01	0.03
	108	–	–	–	–	–	–	0.75

Data are from three replicate experiments.

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immunorecognition caused by fixation in 10% NBF; thus, right-tailed tests also were performed for these data, which also followed the trends observed in the preliminary studies.

Results

Immunostaining scores were more sensitive than the percentage of cells stained. For both DU145 and SKOV3 cells, as the duration of fixation in 10% NBF increased, immunostaining scores decreased so that by 180 h there was no observable PCNA staining (Fig. 1A, B).

After fixation by 10% NBF for 5 min, the PCNA immunostaining scores differed from each subsequent experimental time period for both DU145 and SKOV3 cells (Table 2). A statistically significant decrease ($p < 0.01$) was observed after 18 h in DU145 cells. In SKOV3 cells, a statistically significant decrease ($p < 0.01$) occurred after 36 h.

Immunostaining scores for cells that were fixed for 12 h in 10% NBF followed by 70% ethanol for various periods showed no statistically significant decreases in immunostaining throughout the 180 h of the study for either DU145 or SKOV3 cells (Table 3).

With regard to the percentage of cells stained, there was no statistically significant decrease in staining in either DU145 or SKOV3 cells until after more than 36 h fixation in 10% NBF ($p < 0.01$; Table 4). There were no cells that were stained for PCNA in either cell line after fixation for 180 h. Figure 1C (IV) shows no DU145 cells stained for PCNA after fixation in 10% NBF for 180 h.

There were no statistically significant differences in percentages of DU145 and SKOV3 cell staining for all periods of fixation in 10% NBF for 12 h followed by transfer to 70% ethanol (Table 5).

A statistically significant decrease ($p < 0.01$) in immunostaining scores for all periods was observed for cytokeratins AE1/AE3 in DU145 after fixation for 5 min in 10% NBF compared to other fixation times as shown in Table 6. For SKOV3 cells, a statistically significant decrease ($p < 0.01$) in immunostaining scores was observed after ≥ 12 h fixation in 10% NBF (Table 6). Figure 2A, B demonstrate that in both DU145 and SKOV3 cells, the extent of the decrease in immunostaining scores for cytokeratins AE1/AE3 could be important for interpreting staining after fixation for approximately 36 h in 10% NBF. Figure 2C (IV) shows DU145 cells with significantly reduced staining for cytokeratins AE1/AE3 after fixation for 180 h in 10% NBF.

After fixation for 12 h in 10% NBF and transfer to 70% ethanol, there were no statistically significant changes in immunostaining scores for DU145 or SKOV3 between 12 h and any subsequent times (Table 3).

Compared to fixation for 5 min in 10% NBF, there was a statistically significant decline ($p < 0.01$) in the number of DU145 cells stained for cytokeratins AE1/AE3 after fixation for >18 h in 10% NBF (Table 4); the number of stained SKOV3 cells decreased after 36 h ($p < 0.01$) (Table 4).

There were no significant differences in the percent of DU145 and SKOV3 cells stained for cytokeratins AE1/AE3 after fixation in 10% NBF for 12 h followed by transfer to 70% ethanol (Table 5).

Comparing cytoplasmic immunostaining scores for EGFr in DU145 cells after fixation for 5 min in 10% NBF with other periods of fixation, statistically significant decreases ($p < 0.01$) were observed at

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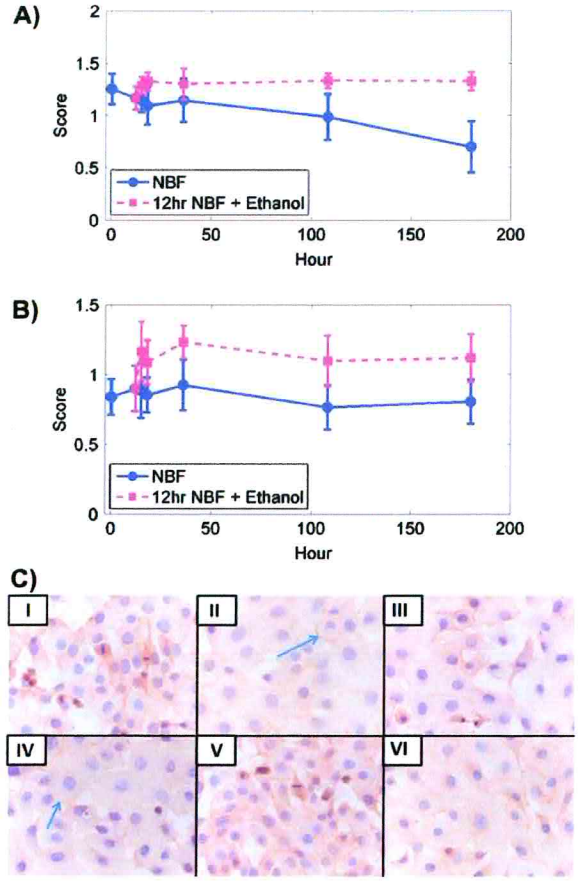

Fig. 4. Membrane immunostaining scores for EGFr. A) DU145 cells after fixation with 10% NBF at times matched with fixation in 10% NBF for 12 h followed by transfer to 70% ethanol at comparable total times of exposure. B) SKOV3 cells after fixation in 10% NBF at times matched with fixation in 10% NBF for 12 h followed by transfer to 70% ethanol for comparable total times of exposure. Data from both cell lines are from three replicate experiments. Error bars are standard deviations. C) DU145 cells after staining for EGFr. I, II, III, IV, V and VI represent 5 min, 12 h, 36 h, and 180 h in 10% NBF, and 12 h in 10% NBF + 24 h in 70% ethanol and 12 h in 10% NBF + 168 h in 70% ethanol, respectively. Blue arrows show membrane staining. × 400.

12 h and subsequent times (Table 7). Although there were statistically significant changes in cytoplasmic immunostaining scores for EGFr in SKOV3 (Table 7), the relative differences were too small to be of practical importance (Fig. 3B).

Cytoplasmic immunostaining scores for EGFr after fixation for 12 h in 10% NBF compared to fixation for 12 h in 10% NBF followed by experimental periods in 70% ethanol showed no statistically significant differences for either DU145 or SKOV3 cells (Table 3).

Comparing the percentages of DU145 cells stained for cytoplasmic EGFr after fixation in 10% NBF for 5 min with other periods, a statistically significant difference was observed only at 180 h exposure to 10% NBF ($p < 0.01$) (Table 4).

The percentage of cells stained for cytoplasmic EGFr in SKOV3 cells after fixation for 5 min in 10% NBF was not statistically different compared to any of the other experimental periods (Table 4).

The percentage of cells with cytoplasmic staining for EGFr after fixation for 12 h in 10% NBF compared to fixation for 12 h in 10% NBF followed by experimental time periods in 70% ethanol showed no statistically significant differences for either DU145 or SKOV3 (Table 5).

Membrane immunostaining scores for EGFr staining in DU145 cells after fixation for 5 min in 10% NBF compared to the subsequent experimental time periods showed statistically significant differences ($p < 0.01$) at 18 h and after ≥ 36 h fixation (Table 8). No statistically significant differences in SKOV3 cells were observed at any of the time intervals. (Table 8) compared to 10% NBF fixation for 5 min.

Membrane immunostaining scores for EGFr for DU145 or SKOV3 cells after fixation for 12 h in 10% NBF followed by transfer to 70% ethanol showed no statistically significant differences at any of the experimental time periods (Fig. 4A, B; Table 3). Figure 4C shows DU145 cells at selected times after fixation by 10% NBF and fixation for 12 h in 10% NBF followed by transfer to 70% ethanol.

The percentage of both DU145 and SKOV3 cells stained were significantly decreased in DU145 cells after 18 h and in SKOV3 cells after 108 h; however, these statistical changes were too small to be of experimental importance (Table 4). Transfer of both DU145 and SKOV3 to 70% ethanol preserved immunorecognition (Table 5).

After fixation in 10% NBF for 5 min, staining of mitoses in DU145 cells with the MIB-1 antibody to Ki67 showed a statistically significant decrease ($p < 0.01$) after 108 or 180 h (Table 8); however, in SKOV3 cells, staining at all subsequent experimental time points were variable (Table 8). Figure 5A, B shows that a significant decrease in MIB-1 staining occurs in DU145 and SKOV3 cells after 108 h and 36 h, respectively, compared to fixation for 12 h in 10% NBF followed by transfer to 70% ethanol for the same amount of time. No statistically significant differences were observed for either DU145 or SKOV3 cells fixed in 10% NBF for 12 h followed by transfer to 70% ethanol for the experimental time periods, (Table 3).

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Table 9. *P*-values of t-tests between any sample pairs of immunostaining for Ki67-MIB-1 in mitotic DU145 cells fixed 10% NBF

	Time in 10% NBF (h)	0.083	12	15	18	36	108	180
DU145	0.083	–	0.12	0.26	0.43	0.48	<0.01	<0.01
	12	–	–	0.68	0.89	0.94	<0.01	<0.01
	15	–	–	–	0.70	0.77	<0.01	<0.01
	18	–	–	–	–	0.58	<0.01	<0.01
	36	–	–	–	–	–	<0.01	<0.01
	108	–	–	–	–	–	–	<0.01
SKOV3	0.083	–	<0.01	<0.01	0.01	<0.01	<0.01	<0.01
	12	–	–	0.83	1.0	0.75	<0.01	<0.01
	15	–	–	–	0.98	0.43	<0.01	<0.01
	18	–	–	–	–	0.02	<0.01	<0.01
	36	–	–	–	–	–	<0.01	<0.01
	108	–	–	–	–	–	–	<0.01

Data are from three replicate experiments.

Discussion

Cells and tissues fixed initially in 10% NBF sometimes may be transferred to 70% ethanol to reduce the loss of immunorecognition that may occur with longer fixation times. Our study demonstrates that this approach may improve immunodetection of some antigens, although the benefits vary with the antigen-antibody pair, and the cell lines and tissues investigated.

We used cell lines grown on coverslips as a model to evaluate the effects of fixation by 10% NBF compared to 10% NBF followed by transfer to 70% ethanol to study the effects of fixation on immunohistochemistry of solid tissue.

Both the proportion of cells stained and immunostaining scores of the cells studied were used to assess the effects of fixation (Grizzle et al. 1998). As expected, the immunostaining score was more sensitive for identifying the effects of fixation on immunorecognition than the percentage of cells stained (Grizzle et al. 1998, Poczatek et al. 1999).

These results agree with previous reports that immunorecognition of specific antibodies of some specific antigens decreases after fixation in 10% NBF and that this decrease is related directly to the duration of fixation (Otali et al. 2009). Frequently, a statistically significant decrease in immunorecognition after fixation for only 12 h in 10% NBF was observed; however, the decrease usually was not large enough to be important for interpretation of staining until after fixation in 10% NBF for 18 h. The extent and time varied with the cell line and antigen-antibody pair. For EGFr, there was less variation in pattern and intensity of immunostaining compared to other antibody-antigen combinations after fixation in 10% NBF or fixation for 12 h in 10% NBF followed by transfer to 70% ethanol.

For all other antigen-antibody pairs studied, transfer from 10% NBF to 70% ethanol after 12 h resulted in improved immunorecognition. The results show the optimal time for transfer to 70% ethanol is between 18 and 36 h of fixation in 10% NBF. This varied slightly with the antigen-antibody pair and the cell line. Our study supports the general approach of transferring cells grown on coverslips, and by analogy tissues, from 10% NBF to 70% ethanol to preserve immunorecognition.

Previously, using a "cell model" of fixation and tissue processing, it was demonstrated that exposure of cells fixed initially by 10% NBF followed by 70% ethanol preserved immunorecognition (Otali et al. 2009). Transfer to 70% ethanol may be useful for immunohistochemistry, because ethanol may facilitate the penetration of antibodies into cells and tissues (Farmilo and Stead 2001). Documented reports on the role that ethanol plays in fixation of cells and tissues and the extent of the effects of ethanol on immunohistochemistry are conflicting. Clearly, 70% ethanol acts as a dehydrating agent for cells and tissues. Some reports indicate that the effects of ethanol on fixation depend on the type of tissue. Ethanol, by extracting lipids, may variably affect tissue antigenicity of specific antigens. For example, in mice, ethanol treatment markedly reduced the detection of Gb_3 in normal kidney tissue, but only minimally in neurons. This variation was attributed to differences in the lipid composition of the tissues (Kolling et al. 2008).

Improved immunohistochemical staining of mammary cancers following fixation in ethanol or alcoholic formalin has been reported for keratins

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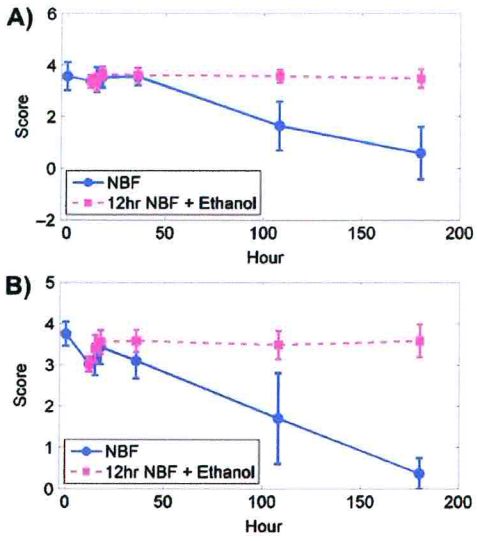

Fig. 5. Ki67-MIB-1 immunostaining scores. A) Immunostaining scores of DU145 cells after fixation in 10% NBF compared to comparable times for 12 h in 10% NBF followed by transfer to 70% ethanol. B) Immunostaining scores for SKOV3 cells after fixation in 10% NBF compared to comparable times for 12 h in 10% NBF followed by 70% ethanol. These data are from three replicate experiments. Error bars are standard deviations.

and p53 compared to fixation with 10% NBF (Arnold et al. 1996). A study that compared the expression of p185erbB-2 in ethanol fixed cell blocks and fine needle aspirates of formalin fixed breast tissue in paraffin blocks reported variable results, although no empirical evidence was presented (Williams et al. 2009).

Fowler et al. (2008) compared the structural properties of RNase A after fixation in 10% formalin, 10% NBF plus ethanol dehydration, or 100% ethanol without prior fixation in 10% NBF. These investigators reported that fixation in 10% NBF for one week did not alter significantly the secondary structure of RNase A. They reported that unfixed RNase A incubated in 100% ethanol for the same period recovered its native structure after ethanol was removed and the RNase A was reconstituted in phosphate buffer. When formaldehyde fixed RNase A was incubated in ethanol for 1 week, then re-hydrated in phosphate buffer, there was a significant decrease in near UV light spectral band intensity, which indicated that the changes were not reversible. Both native and formalin fixed RNase A have been reported to undergo structural transition from the α and β conformation to nearly all β conformation as ethanol concentration was increased from 80 to 100%. Fowler et al. (2008) suggested that exposure to ethanol after formalin fixation causes protein aggregation, which likely stabilizes methylene-bridge crosslinks, hydrogen bonds and van der Waals interactions; this interpretation does not support the preservation of immunorecognition that was observed in these studies.

The permeabilization step during immunostaining required the cells on the coverslips be taken through a series of increasing concentrations of ethanol to acetone, then reversed. This method was used because it gave better immunostaining than other alternatives, e.g., using only acetone or using Triton X-100 and subsequently washing in Tris buffer. This approach of permeabilizing cells (Rodriguez-Burford et al. 2002) is unlikely to have biased experimental observations or interpretations.

Immunorecognition of PCNA, cytokeratins AE1/AE3, and Ki67-MIB-1 decreased significantly after fixation in 10% NBF > 12 h; however, changes in immunorecognition were not large enough to make a practical difference until after exposure to 10% NBF for 18 h. The immunorecognition of EGFr was affected less by fixation in 10% NBF. By contrast, fixation for 12 h with 10% NBF followed by transfer to 70% ethanol between 3 and 168 h prevented significant loss of immunostaining for any of the antigens studied.

Although the results of our study apply most directly to immunostaining of cells, the approach also could be a preliminary model to test whether the immunorecognition by tissues fixed in 10% NBF might benefit from transfer to 70% ethanol prior to processing to paraffin. This study is underway.

To evaluate immunohistochemical staining, the staining score, which considers both the proportion of cells stained and their intensity of staining, is more sensitive than using only the percentage of cells stained for identifying subtle changes in immunostaining as described previously (Poczatek et al. 1999).

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Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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MicroRNAs that affect prostate cancer: emphasis on prostate cancer in African Americans

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Abstract

Although concerted efforts have been directed toward eradicating health disparities in the United States, the disease and mortality rates for African American men still are among the highest in the world. We focus here on the role of microRNAs (miRNAs) in the signaling pathways of androgen receptors and growth factors that promote the progression of prostate cancer to more aggressive disease. We explore also how differential expression of miRNAs contributes to aggressive prostate cancer including that of African Americans.

Key words: African Americans, androgens, growth factors, miRNAs, prostate cancer, receptors

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Prostate cancer (PCa) is the most common tumor among men in the United States. Worldwide epidemiological studies have demonstrated that ethnic origin is an important determinant of PCa risk, incidence and disease progression (DeLancey et al. 2008, Jemal et al. 2010). It has been reported that African American men are more likely to develop PCa at an earlier age, which translates to a 60% greater risk of developing PCa, twice the risk of metastatic disease and greater than twice the PCa associated mortality of Caucasian Americans (DeLancey et al. 2008, Wallner et al. 2009). Many factors, including dietary differences, socio-economic environment, lifestyle and access to adequate medical care have been implicated in the aggressiveness of PCa in African Americans (Sanderson et al. 2004, Williams and Powell 2009); however, these variables do not explain the incidence, aggressiveness and mortality associated with PCa among African Americans. It is Important that differential gene expression and molecular features of PCas in African Americans as contributing variables have not been investigated adequately. Over the past several years, several reports have identified molecular factors that may contribute to the aggressiveness of prostatic neoplastic lesions including those in African Americans.

Altered expression of a host of genes is widely believed to underlie tumor development, progression and metastasis. Although differences in genomic features have been identified, the expression of proteins that ultimately causes tumors to develop and progress is regulated at many levels. Furthermore, identification of molecular mechanisms that regulate critical cellular processes, including cell signaling, cell communication, cell cycle control, cell death, hormonal responses and tumor-cell invasion/metastasis, remain elusive. We focus here on the role of multiple signaling pathways associated with the aggressiveness of PCas that are understood to be influenced strongly by post-transcriptional regulation of prostatic neoplasia by microRNAs (miRNAs).

MiRNAs are small non-coding RNAs that can regulate gene expression post-transcriptionally (reviewed by McNally et al. 2013). Hundreds of miRNAs have been identified as regulators of various molecular processes and each miRNA may regulate the expression of many genes. Cancer type-specific miRNAs can function as oncogenes (oncomiRNAs), tumor suppressor genes, and regulators of metastasis (mestastamiRNAs) (Cho 2007,

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Esquela-Kerscher et al. 2006, Hurst et al. 2009). Few miRNAs have been studied specifically in relation to PCa in African Americans; however, some of the miRNAs and genes that are expressed differentially in PCas of Caucasian Americans likely are important in PCas in African Americans. Also, their actions in African Americans likely are similar to their actions in Caucasian Americans.

The literature concerning the expression of miRNAs in PCas is mixed; many reports do not identify the same pattern of differential expression of miRNAs between PCas and prostate tissues without cancer (Table 1). This likely is due to several factors that include the lack of homogeneity of the malignant tissues and/or the tissues used as controls as well as the choice of controls (Grizzle et al. 2013, McNally et al. 2013). For example, in many tissues, both malignant and nonmalignant, extensive infiltration by inflammatory cells may occur. Inflammatory cells can produce miRNAs and these may contribute to overall miRNA in the tissues; this could result, for example, in the miRNAs from inflammatory cells being incorrectly attributed to malignant cells, i.e., a false positive result. Uninvolved nonmalignant prostatic glands and stroma also frequently contaminate samples of PCa, or alternatively, neoplastic cells may contaminate control samples of the prostate if controls are obtained from tumor-free regions of prostates with PCa; such factors decrease the clarity of assessment or may cause false positive results. As demonstrated in Fig. 1, infiltrating cancer cells may be mixed with the glands of BPH or PCa. Similarly, uninvolved glands may be mixed with cancer and cancer may be mixed with uninvolved glands.

The results for some miRNAs may vary depending on the preparation of the tissue specimens (e.g., frozen vs. paraffin embedded) or whether data are obtained and reported using only cell lines. The literature indicates that miRNAs in PCa may vary depending on subcategories of PCa, e.g., aggressive vs. non-aggressive, primary vs. metastatic lesions, hormonally responsive PCa vs. hormonally resistant, variations among races and mixtures of these categories (Porkka et al. 2007, Tong et al. 2009, Szczyrba et al. 2010, Watahiki et al. 2011). The molecular features of uninvolved prostate glands also may be influenced by the presence of adjacent tumor (Gaston et al. 2012). Specific molecular subtypes of prostatic adenocarcinomas, e.g., PCas with mutations of p53 and/or with gene fusions such as TMPRESS2:ERG, may produce different patterns of expression of miRNAs. An example of this is miRNA-21, which has been reported by some to be up-regulated in PCa (Volinia et al. 2006, Szczyrba et al. 2010), but not reported by others to be expressed differentially in PCa (Folini et al. 2010). This contradiction may be explained by the reported miRNA-21 suppression of the protein, reversion-inducing cysteine-rich protein with Kazal motifs (RECK), in PCa. While Folini et al. (2010) and Reis et al. (2012) found miRNA-21 to be decreased in the majority of cases of PCa, the latter investigators found that it was selectively overexpressed and RECK, and under-expressed in higher stage (pT_3) lesions, especially in metastatic lesions (Watahiki et al. 2011). It is possible also that experimental errors or incorrect interpretations contribute to variable results.

Table 1. Examples of miRNAs reported to be inconsistently expressed differentially (different directions)

MiRNA	References to reports of down-regulation of miRNA in prostate adenocarcinomas (fold change if specified)	References to reports of up-regulation of miRNA in prostate adenocarcinomas (fold change if specified)
Let-7c	Nadiminty et al. 2012 Ozen et al. 2008 Porkka et al. 2007	Szczyrba et al. 2010 (2.7)
miRNA-26b	Porkka et al. 2007[1]	Szczyrba et al. 2010 (1.7)
miRNA-125b	Szczyrba et al. 2010 (−1.5) Ozen et al. 2008	Shi et al. 2007
miRNA-126	Carlsson et al.	Szczyrbra et al. 2010 (1.9)
miRNA-223	Szczyrba et al. 2010	Volinia et al. 2006

[1]Only in hormone resistant cases

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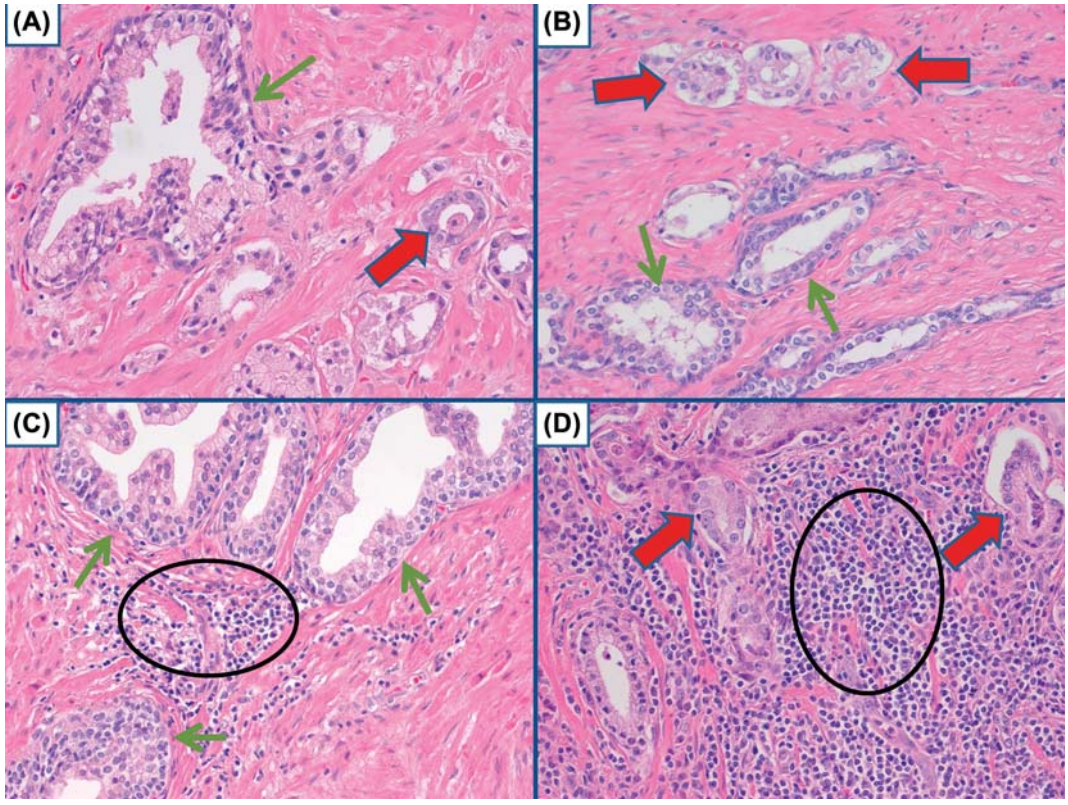

Fig. 1. Various mixtures of cell types. A) Benign prostate gland (green arrow) with adjacent PCa (red arrow). B) PCa (red arrows) adjacent to benign prostate glands (green arrows). C) Benign prostate glands (green arrows) with intermixed inflammatory cells, mostly lymphocytes (black circle). D) PCa (red arrows) surrounded by inflammatory cells that primarily are lymphocytes (black circle). Hematoxylin and eosin. x 200.

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Our review emphasizes the miRNAs that may be subtype dependent, e.g., androgen refractory PCa, as well as miRNAs that have been reported more than once to be involved in PCa and/or to be relevant to PCas in African Americans. We emphasize miRNAs and genes that contribute to aggressive PCa, especially in African Americans, and the role, or putative role, of miRNAs in regulating genes that affect progression and metastasis of PCa.

MiRNAs considered to be regulated differentially in PCas

Several studies have reported that miRNAs are involved in the development and progression of PCas. Specifically, miRNAs are known to be expressed differentially in PCas compared to benign prostatic tissues (Table 2).

When PCa occurs, it is treated first using methods to reduce androgens or the effects of androgens; however, most tumors ultimately become resistant to the withdrawal of androgens. Several miRNAs have been associated with the effects of androgens or modulation of androgen receptors. Examples of such miRNAs are listed in Table 3.

MiRNAs appear to play an important role in the progression of PCa to metastatic disease. In a study of miRNAs expressed differentially in a metastatic PCa cell line developed using xenografts, Watahiki et al. (2011) identified 23 miRNAs that were up-regulated more than fivefold in the metastatic line compared to the matching non-metastatic cell line and 24 miRNAs that were down-regulated more than fivefold in the metastatic line. Of the down-regulated miRNAs, miRNA-205, miRNA-503, miRNA-708 and miRNA-2115 that were detected in the non-metastatic cell line, were undetected in the metastatic line. In the metastatic cell line, miRNA-21 was expressed most strongly and miR-148 was expressed most strongly in the non-metastatic cell line. Also, miRNAs from different DNA arms, e.g., 5p and 3p, sometimes showed different expressions between metastatic and non-metastatic subgroups as well as miRNAs with very small differences between their sequences. For example, miRNA-126* was down-regulated in the

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Table 2. Examples of miRNAs reported more consistently to be expressed differentially in prostate adenocarcinomas

MiRNA	References to consistent reports of down-regulation of miRNA in prostate adenocarcinomas (fold change if specified)	References to consistent reports of up-regulation of miRNA in prostate adenocarcinomas (fold change if specified)
miRNA-20a		Szczyrba et al. 2010 (1.5) Volinia et al. 2005
miRNA-23b	Tong et al. 2008 (−1.2) Porkka et al. 2007[1]	
miRNA-25		Szczyrba et al. 2010 (1.8) Volinia et al. 2006 Ambs et al. 2008
miRNA-26a	Carlsson et al. 2011 Porkka et al. 2007	
miRNA-27b	Carlsson et al. 2011 Szczyrba et al. 2010 Porkka et al. 2007[1]	
miRNA-29a	Volinia et al. 2005 Porkka et al. 2007[1] Szczyrba et al. 2010 (−2.4) Ru et al. 2012	
	Porkka et al. 2007[1] Steele et al. 2010	
miRNA-32		Volinia et al. 2006 Ambs et al. 2008
miRNA-100	Tong et al. 2008 (−1.2) Porkka et al. 2007[1]	
miRNA-106a		Szczyrba et al. 2010 (2.8) Volinia et al. 2006
miRNA-106b		Szczyrba et al. 2010 (2.3)
miRNA-143	Szczyrba et al. 2010 (−4) Porkka et al. 2007 Carlsson et al. 2011 Tong et al. 2008 (−1.3)	
miRNA-145	Zaman et al. 2010 Porrka et al. 2007 Ozen et al. 2008	
miRNA-205	Hulf et al. 2012 Porkka et al. 2007[1]	
miRNA-221	Tong et al. 2008 (−1.3) Porkka et al. 2007	
miRNA-222	Tong et al. 2008 (−1.5) Porkka et al. 2007	

[1]Only in hormone resistant cases.

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Table 3. MiRNAs associated with hormonally resistant prostate cancer

MiRNA	Reference to down-regulation in hormonally resistant prostate cancer	Reference to up-regulation in hormonally resistant prostate cancer
miRNA-100	Porkka et al. 2007	
miRNA-124	Shi et al. 2012	
miRNA-125b	Shi et al. 2007[1] Porkka et al. 2007[2]	
miRNA-146a	Xu et al. 2012 Li et al. 2008	
miRNA-148a	Porkka et al. 2007	
miRNA-198		Porkka et al. 2007
miRNA-205	Porkka et al. 2007	
miRNA-221	Sun et al. 2009	
miRNA-222	Sun et al. 2009	
miRNA-345		Porkka et al. 2007
miRNA-448		
miRNA-616		Ma et al. 2011

[1]Cell line
[2]All PCa

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metastatic cell line (3.4 fold change), but miRNA-126 was increased in the metastatic cell line (14 fold change). Selected results from the study of Watahiki et al. (2011) and other studies are included in Table 4.

Several of the miRNAs associated with differential expression in metastatic PCa have been associated with genes involved in metastasis. For example, miRNA-205 reduces the protein expression of ZEB1, which is involved in epithelial to mesenchymal transition (EMT), and p63 acts by stimulating miRNA-205 to inhibit ZEB1 and therefore EMT (Tucci et al. 2012). Similarly, miRNA-203 targets and inhibits ZEB2 and other miRNAs involved in metastasis including Runx2, a major participant in bone metastases, and survivin, poly-comb repressor, Bmi and SMAD4 (Saini et al. 2011).

MiRNAs expressed differentially in African American prostate tumors

Calin and Croce (2006) reported that miRNA-1b-1, miRNA-26a and miRNA-30c-1, miRNA-219, and miRNA-301 are expressed differentially in PCas from African Americans compared to those from Caucasian Americans (Calin and Croce 2006). We extended this study using cultures of the novel African American PCa cell lines, RC77N/E, RC77T/E and MDA-2Pca-2b. The expression of miRNA-26a in these cell lines was compared to its expression in the Caucasian American derived cell lines, PrEC, RC-92a and PC-3 (Theodore et al. 2010a,b). Comparing African American to Caucasian American derived cell lines derived from tumors of the same grade and stage, we found a 2.3 fold increase of miRNA26a in nonmalignant cell lines, a 13.4 fold increase in malignant cell lines and 2.4 fold increase in metastatic cell lines. PCa cell lines from African Americans showed the greatest expression of miRNA-26a among all cell lines tested. Our unpublished data indicate that after only 10 min, miRNA-26a is responsive to EGF stimulation and depletion of miRNA-26a induces G2/M arrest and subsequent activation of caspase 3/7. Thus, the increased incidence of PCa associated with the African American race may be associated with regulation of genes associated with apoptosis. For example, Bcl-2, an anti-apoptotic protein and a miRNA-26a target, has altered expression in African American PCa (Guo et al. 2000).

The expression of miRNA-151 in African American men after radical prostatectomy with undetectable postoperative prostate specific antigen has been compared with miRNA-151 expression in African Americans who had radiation or androgen ablation therapy followed by rising prostate specific antigen levels. MiR-151 was increased and the NKX3-1 gene, which it regulates, decreased in 67% of African American patients with rising PSA who were treated with radiation or androgen ablation compared to only 17% of African American patients without increasing PSA who were treated with radical prostatectomy (Barnabas et al. 2011). Similarly, miRNA151 expression has been reported to be correlated with the aggressive characteristics of hepatocellular carcinoma cell lines (Ding et al. 2010). Thus, miRNA-151 eventually might be useful for identifying aggressive PCa in African American men.

MiRNAs, androgens, androgen receptors and race

PCa growth depends on testosterone and its active metabolite, 5α-dihydrotestosterone (DHT). Levels of testosterone and DHT differ among different ethnic groups; both male and female African Americans

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Table 4. Examples of miRNAs expressed differentially in metastatic prostate cancer and also identified by other studies (based primarily on Volinia et al. 2006, Porkka et al. 2007, Tong et al. 2009, Szczyrba et al. 2010, Watahiki et al. 2011, Carlsson et al. 2011)

MiRNA	Down-regulated in metastatic cell line (fold change if specified)	Previously reported to be differentially expressed in prostate cancer compared to benign prostatic tissue (fold change if specified)	Previously identified as modulating metastasis of prostate cancer	Previously reported as affecting metastasis in a different cancer (e.g., breast cancer)
miRNA-16	(−20)	Down-regulated[2]	Yes	Yes
miRNA-24	(−8)	(−1.5)		Yes
miRNA-29a	(−30)	(−2)		
miRNA-34a	(−3)	Down-regulated[2]	Yes	Yes
miRNA-126*	(−3)	Down-regulated[2]		
miRNA-145	(−3)	(−4)	Yes	Yes
miRNA-195	(−5)	Down-regulated[2]		
miRNA-203	(−1.5)	Down-regulated[2]	Yes	Yes
miRNA-205	(NA)[1]	Down-regulated[2]	Yes	Yes
miRNA-221	Down-regulated[2]	(−1.3)(−2)	Yes*	**
miRNA-425	(−6)	Down-regulated[2]		
miRNA	**Up-regulated in metastatic cell line (fold change if specified)**	**Previously reported to be differentially expressed in prostate cancer compared to benign prostatic tissue (fold change if specified)**	**Previously identified as modulating metastasis of prostate cancer**	**Previously reported as affecting metastasis in a different cancer (e.g., breast cancer)**
Let7i	(2)	Up-regulated[2]		Yes
miRNA-21	(1.7)	Variable reports		Yes
miRNA-106a	(3.3)	(2.8)		Yes

[1]Cannot be calculated because it was not detectible in metastatic tumors
[2]Extent of differences in expression
*Correlated with TMPRESS2:ERG fusion (Gordanpour et al. 2011)
**Up-regulated in aggressive breast cancer and hepatocellular carcinoma (Shah and Calin 2011, Fu et al. 2010)

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show greater levels of both hormones from birth through adolescence than Caucasian Americans (Abdelrahaman et al. 2005, Joseph et al. 2002, Winters et al. 2001).

The enzyme, 5-α-reductase type 2, which converts testosterone into DHT, may cause elevated levels of DHT in African Americans, because the activity of the gene, SRD5A2, which codes for this enzyme, is elevated in African Americans (Morissette et al. 1996, Litman et al. 2006, Thomas et al. 2008). Also, genetic variations of the SRD5A2 gene in African American men, such as TA repeat alleles and mis-sense mutations (A49T variant), cause increased activity of 5-α-reductase (Giwercman et al. 2005), which is correlated with increased risk of PCa (Kubricht et al. 1999, Li et al. 2011). In addition, over-expression of 5-α-reductase type 1 causes increased sensitivity to androgens in PCa cells (Thomas et al. 2009).

African American men have fewer G and GGC repeats in the androgen receptor gene than Caucasian American men (Bennett et al. 2002, Irvine et al. 1995, Platz et al. 2000, Sartor et al. 1999); this is correlated with a more active androgen receptor and increased sensitivity to circulating androgens (Beilin et al. 2000, Chamberlain et al. 1994). Increased androgenic activity has been demonstrated in both benign and malignant prostatic tissue in African American men and may explain why African Americans with PCa have lower biochemical failure rates after androgen deprivation therapy (androgen deprivation therapy) than Caucasian Americans (Gaston et al. 2003). It is interesting that African Americans with metastatic PCa whose tumors have become androgen resistant (characteristic of the phenotype that develops under conditions of low androgen receptor levels) exhibit poorer responses to chemotherapy, poorer therapeutic outcomes, and poorer quality of life than comparable Caucasian American patients (Thatai et al. 2004).

As a transcription factor, androgen receptor regulates positively and negatively the expression of hundreds of both coding and noncoding RNA targets, including miRNA-125b, which is up-regulated by androgens. MiRNA-125b suppresses Bak1 and induces androgen-independent growth

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of LNCaP and the subline, cda1 LNCaP (Shi et al. 2007). Similarly, it has been reported that miRNAs are involved in the androgen receptor signaling pathway. Ribas et al. (2009) demonstrated that androgen receptor binds to the promoter of miRNA-21, which in turn can stimulate both hormone-dependent and -independent PCa growth. Forced expression of miRNA-21 enhanced PCa growth in vivo and enabled androgen-dependent cancer cell lines to overcome castration-mediated growth arrest (Ribas et al. 2009). MiRNA-21 has been reported to be associated with high stage and metastatic PCas (Reis et al. 2012, Li et al. 2012) and serum levels of miRNA-21 have been reported to be elevated in patients with metastatic hormone-refractory PCa (Li et al. 2011, Zhang et al. 2011, Agaoglu et al. 2011). Agaoglu et al. (2011) also reported higher levels of miRNA-221 in the blood of patients with PCa. Although it has not been determined whether there is racially associated expression of miRNA-21, this miRNA could provide valuable insight into the progression of aggressive PCas.

Östling et al. (2011) reported that miRNA-9, miRNA-34a, miRNA-34c, miRNA-135b, miRNA-185, miRNA-297, miRNA-299-3p, miRNA-371-3p, miRNA-421, miRNA-449a, miRNA-449b, miRNA-634, and miRNA-654-5p can bind directly to the 6kB extended arm of the 3′ UTR of androgen receptor. The majority of these miRNAs regulate androgen receptor primarily through the extended arm region, and miRNA-34a and miRNA-34c correlate negatively with androgen receptor expression levels in clinical cases of PCa (Östling et al. 2011). MiRNA-34a also was demonstrated to be a key regulator of the CD44 positive putative stem cell population in PCas (Östling et al. 2011, Liu et al. 2011). Given the important role of cancer stem cells (CSC) in development of PCa, miRNA-34a could be a novel therapeutic target for PCa (Fujita et al. 2008).

The expression and activity of androgen receptor plays not only a central role in development of PCa, but also is important for the development of castration-resistant or hormonally resistant PCa (HRPC). The let 7 family of miRNA's have been shown to play a pivotal role in PCa. Let-7c is a key suppressor of androgen receptor expression by targeting c-Myc. (Chang et al. 2009). Down-regulation of let-7c in PCa specimens is correlated inversely with androgen receptor expression, while the expression of Lin-28, a repressor of let-7, is correlated positively with androgen receptor expression. Furthermore, suppression of androgen receptor by let-7c causes decreased proliferation of PCa cell lines (Nadiminty et al. 2010) and miRNA-146a behaves similarly by targeting the androgen receptor directly (Lin et al. 2008). The miRNAs that affect or are affected by androgens including let7c, miRNA-34a, miRNA-34c, and miRNA-146a have not been evaluated directly in PCa of African Americans, but given the clear contribution of androgen receptor signaling to aggressive PCa in African American men, more investigation in this area is warranted.

Although once viewed as separate events in the development and progression of PCa, Sun et al. (2012) found a relationship between epithelial to mesenchymal transition (EMT) and androgen-deprivation therapy; this is discussed below.

MiRNAs and growth factor expression

The growth factors and their receptors that have been reported to be involved in PCa include members of the epidermal growth factor (EGF), scatter factor/hepatocyte growth factor (SF/HGF), transforming growth factor beta (TGFβ) and basic fibroblast growth factor (bFGF) families. These growth factors are responsible for modulating cellular differentiation, migration, proliferation and cell death, e.g., apoptosis. EGFR has been implicated in epithelial cell malignant transformation in most PCa cell lines in which androgen-independent cells, e.g., DU145, express more EGFR than the androgen dependent cell lines (De Miguel et al. 1999, Kim et al. 1999, Turner et al. 1996). Membrane-specific EGFR has been reported to be over-expressed in prostate tumors from African Americans and to be correlated with higher pre-treatment levels of prostate specific antigen and higher stage tumors (Shuch et al. 2004, Douglas et al. 2006). Douglas et al. (2006) also reported four novel mis-sense mutations in exons 19, 20 and 21 of the EGFR tyrosine kinase domain in Koreans and Caucasian Americans, but none of these mutations was found in African Americans (Douglas et al. 2006). It is interesting that in hormonally resistant PCa, miRNA-146a, binds to the 3′-UTR of the mRNA of EGFR and inhibits its downstream signaling, e.g., pERK1/2 and MMP2. The expression of miRNA-146a was decreased in HRPC compared to androgen-dependent PCa (ADPC)(Li et al. 2008, Xu et al. 2012). This correlates with increased expression of EGFR in DU145 cells. Similarly, miRNA-146a down-regulates EGFR in pancreatic cancer (Li et al. 2010). MiRNA-132, which targets heparin-binding epidermal growth factor and TALIN2, is thought to control cellular adhesion and to play a role in suppressing metastases in PCa (Formosa et al. 2012).

In PCa, autocrine signaling by EGFR leads to dysregulation of various signaling cascades that

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have been shown to be dysregulated in PCa in African Americans. For example, over-expression of EGFR associated Son of Sevenless homolog 1 (SOS1), activator of Ras/MAPK and downstream MMP proteins, have been linked with PCa in African Americans (Hatcher et al. 2009, Shuch et al. 2004, Timofeeva et al. 2009). Recently, we demonstrated that Kaiso, a BTB-POZ methylation binding protein, is up-regulated in late stage prostate tumors, and this is the result of EGFR signaling (Jones et al. 2012). In addition to EGFR, TGFα, HER2 and HER3 also are expressed in PCa (Myers et al. 1994, 1995). MiRNA-331-3p binds to the 3′ UTR of the mRNA for HER2, inhibits the expression of HER2 and indirectly reduces androgen receptor signaling by decreasing HER2 (Epis et al. 2009).

Androgen deprivation therapy has been shown to enhance EMT in LnCaP cells and to promote chemoresistance by ZEB1, which mediates androgen deprivation-induced EMT by a bi-directional, negative feedback loop including miRNA-200. Expression of ZEB1 and ZEB2, which facilitate development of EMT, are regulated negatively by five members of the miRNA-200 family (miRNA-141, miRNA-200a, miRNA-200b, miRNA-200c, and miRNA-429), which are highly homologous. These miRNAs have been reported to bind directly to the 3′ UTR of ZEB1 and to cause degradation of its mRNA, which results in up-regulation of E-cadherin in cell lines of several cancers (Burk et al. 2008, Wellner et al. 2009, Gregory et al. 2011).

Several reports have shown that EGFR and platelet-derived growth factor receptor (PDGF-R) influence the expression of the miRNA-200 family. By their regulation of the EMT process, the miRNA-200 family affects sensitivity to therapy focused on EGFR and its signal transduction pathway in cell lines of several cancers including those of the bladder, breast, lung and pancreas. In general, there are both autocrine and paracrine signaling networks that involve TGFβ and the miRNA-200 family; a ZEB/MiR-200 balance shifts cells between epithelial and mesenchymal phenotypes (Gregory et al. 2011). The re-expression of miRNA-200s is sufficient to reverse the mesenchymal phenotype to an epithelial profile (Korpal et al. 2008) and to re-establish EGFR dependency in several cell lines of various cancers. ZEB1 also represses the epithelial phenotype in PCa cells and therefore facilitates cellular transendothelial migration (Drake et al. 2009).

Platelet-derived growth factor-D (PDGF-D) has been shown to induce EMT in PC-3 cells and to decrease the expression of members of the miRNA-200 family. Specifically, PDGF-D induces EMT by up-regulation of ZEB1, ZEB2 and Snail by down-regulation of miRNA-200a, miRNA-200b and miRNA-200c. TGFβ increases the expression of ZEBs and DNA methylation of miRNA-200s, while miRNA-200s inhibit ZEBs (Gregory et al. 2011). When miRNA-200b was re-expressed in PC-3 cells, ZEB1, ZEB2, and Snail decreased and this decrease caused a reversal of EMT; EMT has been associated with decreased cellular migration and invasion as well as changes in cancer stem-like cells (Kong et al. 2008, 2009, 2010). In breast cancer, miRNA-221/222 opposes the actions of the miRNA-200s (Howe et al. 2012), so the effects of the miRNA-200s in PCa might be reversed by miRNA-221/222. Confirmation of this in PCa would be useful for understanding how the actions of specific miRNAs can be generalized. The interactions between the miRNA-200 family and ZEB1, ZEB2 and Snail have been reported for several types of tumors, so this seems to be a general feature of EMT regulation of several types of cancer. MiRNA-203 and miRNA-205 also are likely to regulate EMT (Tucci et al. 2012, Vandenabeele et al. 2012, Saini et al. 2011).

In addition to EGFR and PDGF-R, activation of IGFR is involved in modulating EMT. High serum levels of insulin-like growth factor-I (IGF-1) and lower levels of IGF binding protein-3 (IGFBP-3) have been shown to be associated with increased risk of PCa (Chan et al. 2000, Hernandez et al. 2007, Tricoli et al. 1999). In a cohort of 401 patients with PCa and 366 age, sex and ethnicity matched controls, it was found that variations in the 5′-untranslated region of both the IGF-1 and IGFBP-3 genes may have increased IGF serum levels and that the increased levels were correlated with PCa in African Americans (Hernandez et al. 2007, Yates et al. 2005, 2007). As with EGFR, we and others have demonstrated that activation of IGFR-1 is responsible for the loss of cell-cell adhesion, which leads to EMT promotion of metastasis (Graham et al. 2008, Yates et al. 2007) by increased Snail, ZEB1 and ZEB2 (Graham et al. 2008). Interestingly, in metastatic pancreatic cancer cell lines, IGF-R1 is regulated positively by miRNA-100; both miRNA-100 and IGF-R1 are increased in pancreatic cancer cell lines that have the potential to metastasize, but not in pancreatic cancer cell lines that do not have the potential to metastasize (Huang et al. 2013).

MiRNAs and regulation of oncogenes, suppressor genes and other genes that affect PCa

Several miRNAs may affect the growth and progression of PCa by interactions with oncogenes,

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Table 5. Actions of miRNA on oncogenes and suppressor genes in prostate cancer

MiRNA	MiRNAs down-regulated by hypermethylation of their control elements	MiRNAs with hypomethylated control elements
miRNA-34a	Kong et al. 2012	
miRNA-145	Suh et al. 2011	
miRNA-124	Lujambio et al. 2007 Shi et al. 2012	
miRNA-196b	Hulf et al. 2011	
miRNA-126	Saito et al. 2009	
miRNA-200c	Vrba et al. 2010	
miNAR-141	Vrba et al. 2010	
miRNA-615		Hulf et al. 2011

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suppressor genes and genes involved in progression and metastasis. For example, miRNA-221 and miRNA-222 target the mRNA of the suppressor gene, androgen receptor, ARHI (Chen et al. 2011); it is interesting that genistein down-regulates miRNA-221 and miRNA-222 and by this pathway decreases androgen receptor, ARHI. MiRNA-221 and miRNA-222 also down-regulate the androgen-sensitive cell cycle inhibitor, $p27^{kip-1}$ (Myers et al. 1999, Sun et al. 2009). Similarly, by analogy to breast cancer, miRNA-221 and miRNA-222 might counter the effects of the miRNA-200 family on EMT (Howe et al. 2012). The targets of the miRNA-200 family also include ERRFI-1, which is a novel regulator of EGFR-independent growth. Similarly, the protein, high motility group AT-hockgene 1(HMGA1), is a nuclear binding protein that has oncogenic properties by facilitating chromosomal instability. HMGA1 is increased in high grade and advanced stage PCa. The expression of HMGA1 is inhibited by miRNA-296, which acts with HMGA1 to inhibit proliferation (Wei et al. 2011). It is noteworthy that the expressions of miRNA-1 and miRNA-133a are decreased in PCa and that these inhibit purine nucleoside phosphorylase (PNP), which facilitates proliferation, migration and invasion of the PC3 and DU145 PCa cell lines. Thus, miRNA-1 and miRNA-133a act as suppressor genes.

MiRNA-616 induces androgen independent growth by targeting tissue factor pathway inhibitor 2 (TFPI-2). MiRNA-101 may reduce the growth of PCa by inhibiting the expression of Cox2 by targeting the 3′ untranslated region (3′-UTR) of the mRNA of Cox2 (Hao et al. 2011).

MiRNAs and epigenetic control of PCa

Epigenetic control of PCa involves specific miRNAs that are targets, i.e., epigenetically regulated (Table 5), and/or some miRNAs directly affect enzymes and other molecules involved in epigenetic control (Paone et al. 2011). Examples of miRNAs that modulate epigenetic control in PCa include miRNA-101, which inhibits the oncogenetic protein enhancer of zeste homolog 2 (EZH2) and miRNA-449a, which down-regulates histone deacetylase 1 (Varambally et. al. 2008, Cao et al. 2010, Noonan et al. 2009). Similarly, miRNA-34b down-regulates DNA methyl transferases and histone deacetylases (Majid et al. 2012). Regulation of miRNAs by methylation of CpG islands in their promoters may be quite variable in PCa. For example, only 42% of PCas, which had methylated promoters for miRNA-132 were also correlated with both Gleason scores and stages of the tumors (Formosa et al. 2012).

MiRNA therapy for PCa

Because of the involvement of miRNAs in regulating the androgen receptor, it would be logical to expect that targeting selected miRNAs or up-regulation of specific miRNAs might be important for androgen deprivation therapy. The development of "antagomiRNAs," which are small oligonucleotides chemically engineered to target small nucleotide regions of specific miRNAs to selectively silence the targeted miRNA, should lead to approaches that make androgen deprivation therapy more effective initially and that extend the response of PCa to androgen deprivation therapy (Krützfeldt et al. 2005). Similarly, the involvement of miRNAs in modulating responses to chemotherapeutic drugs could be important for therapies for PCa.

When PCas fail androgen deprivation therapy, chemotherapy is used primarily for palliative therapy and to extend survival. Thus, it would be interesting to determine whether modulating specific miRNAs might make chemotherapeutic agents

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more effective and reduce and/or retard the development of chemoresistance to specific drugs. In this regard, the ectopic expression of miRNA-21 has been associated with resistance to docetaxel in PC3 cells (Shi et al. 2010). By contrast, ectopic expression of miRNA-34a decreases the phenotypic expression of the protein, silent mating type information regulation 2/homolog 1 (*S. conevisiae*) (SIRT1) as well as Bcl-2 and HUR., These changes enhanced the chemosensitivity of PC3 cells to camptothecin (Fajita et al. 2008) and PC3 paclitaxel resistant cells (PC3PR) to paclitaxel (Kojima et al. 2010). Also, chemoresistance may be induced by miRNA stimulation of specific pathways such as hedgehog (Singh et al. 2012). In addition, miRNAs such as miRNA-210 modulate a stress response to therapeutic approaches (Cui et al. 2012). These and similar studies suggest that miRNAs are involved in both chemosensitivity and development of chemoresistance, hence their modulation may improve therapy for PCa.

Several signaling pathways appear to be dysregulated at the post-transcriptional level in PCa. The involvement of miRNAs in molecular regulation of genes and underlying tumor biology could explain the aggressiveness observed in some PCa; however, more insight into the post-transcriptional regulation of the genes involved in the development and progression of PCa is needed before miRNAs can be used for therapeutic targeting and as biomarkers. There are a few reports that regulation of gene expression by miRNAs contributes to aggressive disease in African American patients. More work is needed to explore miRNA mediated gene regulation that affects the African American population disproportionally.

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Human Tissue Biorepository

17

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17.1 Models of Human Tissue Repositories

Human Tissue Biorepository – an organization that is involved in any aspect of collecting, processing, storing, banking, or distributing human tissues for research, education, or clinical uses. This chapter focuses only on human tissue biorepositories supporting research.

- "Catch as catch can" – disorganized approach to collection, processing, storage, and distribution. The repository collection may not be very useful to support research
- Banking model – general tissues or specific tissues are collected according to a standard operating procedure (SOP)
  - Emphasis is usually on banking rather than distribution
  - Tissues are immediately available
  - Outcome and clinical information may be immediately available
  - Fresh specimens usually are not available
  - Increased storage requirements
  - Specimens may not meet investigator needs
  - Specimens may not be used
- Clinical trial model – subtype of banking model in which remnant tissues from clinical trials are stored
  - Specimens usually consist of cases and controls, both of which have extensive clinical information and outcome
  - Some specimens may not be able to be used for general research due to limitations of informed consent
  - Some specimens may not meet investigator needs
  - Some specimens may not be used
- Prospective model – human tissue specimens are collected to specifically meet each investigator's specific requests
  - Specimens are collected with SOPs which are established by the investigator
  - Most specimens are used, so operation is very efficient and cost effective
  - Decreased storage requirements
  - Fresh specimens are available
  - Specimens are collected prospectively so they are not immediately available when requested
  - Clinical outcome and other data are not available when specimens are requested. Outcome may take many years depending upon the disease process
- Combination of banking and prospective models – best of both approaches

17.2 Aids in Developing Tissue Repositories

- Best practices
  - International Society of Biological and Environmental Repositories (ISBER), third edition has been published in Biopreservation and Tissue Banking
  - National Cancer Institute, Best Practices, second edition
- Comprehensive and special references on repository operations
- General issues to be considered in development and operations of a human tissue biorepository
  - Quality assurance (QA) in operations
    - All operations based on a QA program
    - Quality control of tissue specimens
  - Repository science – knowledge of factors affecting quality of tissues provided to support research
  - Safety
    - Repository personnel
    - Investigators and their personnel
  - Regulations and ethics, IRBs, privacy board
    - Repositories
      - Institutional Review Board (IRB) approves repository – usually requires full review
      - Repository meets Health Insurance Portability and Accountability Act (HIPAA) requirements for protected health care information and standards for security of informatics program
    - Investigators
      - IRB may classify study as nonhuman research
      - IRB may classify study as exempt

  - IRB may approve study
- HIPAA may not apply if
  - None of 18 personal identifiers as defined by HIPAA are utilized in patient data
  - Limited dataset, which includes dates, can be used with a research agreement
- IRB may classify study as nonhuman research
  - De-identified cases (coded) – investigators have no access to the identities of tissue sources
  - Anonymized – neither repository nor investigator has access to identities of tissue sources
- IRB classifies study as exempt based on de-identified or anonymized tissue specimens
- IRB approves study – expedited or full review

17.3 Design of Repository

- Goals
  - Who are the customers and what do these investigators want?
    - What do they need?
    - What is available?
  - What types of tissues (e.g., frozen tissue) to collect and provide?
  - What is the model of a repository which will be used?
  - What not to collect?
    - Consider if specimens infected with blood-borne pathogens (e.g., HIV) will be collected
  - What not to do – do not perform the investigators research for them
- Resources to support the repository (see Box 17.1)

Box 17.1. Resources to support the repository

- Initial resources available
  - Space
  - Equipment
  - Funds
    - Personnel
- Long-term support
  - Long-term commitment – is it worth the effort to start the repository?
  - Cost recovery for all work performed for investigators

17.4 Challenges in Collecting Specimens

- Identification of cases of interest
  - Collecting bodily fluids at clinic visit prior to the operation
    - Fasting versus nonfasting
    - Efficiency of collection
    - Avoiding a separate blood draw
  - Operative schedules to identify cases of interest
  - Distributing sterile collecting containers to operating room (OR)
  - Rapid transfer from OR to pathology for diagnostic review
  - Obtaining specimens not needed for diagnosis directly from pathology
- Limitations to collection of specimens
  - Neoadjuvant therapy – therapy that occurs prior to surgery so that the tissue is "affected" before being collected
    - Population of cells may be lost from the tumor or the tumor and/or metastases may be destroyed
    - This is a great challenge to tissue biorepositories independent of the model
  - Size that requires all specimens to be submitted diagnostically
    - More effective screening/detection/diagnosis is resulting in smaller tissue specimens, especially tumors
  - Preinvasive neoplastic specimens (e.g., ductal carcinoma in situ of the breast) completely submitted to identify any areas of invasive disease

  - Newer approaches to diagnosis
    - Imaging rather than biopsy to identify metastases and smaller tumors
    - Needle aspirations to identify larger tumors and metastasis resulting in no excess tissues being available to support research
  - Warm ischemia during surgery affecting tissue available for research
- When surgery is undertaken, blood vessels are completely or partially compromised and a period described as warm (body temperature) ischemia may affect specimens
  - Ischemia causes loss of rapidly degraded molecules including mRNAs and phosphoproteins
  - Blood vessels usually are not compromised when biopsies are obtained
  - There may be differences between rapidly degraded molecules in biopsies versus resections
  - Because many rapidly degraded molecules have been lost during warm ischemia, remaining molecules seem much more stable when tissue removed from body
  - Time for collection/processing – speed the processing to limit cold ischemia
- After the removal of tumors from body, changes may occur less rapidly than changes with the body during ischemia
  - Resources may limit ability to rapidly process specimens
  - Flow of specimens from the operating room (OR) should be considered
  - Pathology administrative requirements (e.g., when to enter the specimen into clinical informatics system)
  - Quickly processed aliquots versus longer processed aliquots from same tissues
  - Keep specimen at 4 °C until processed
  - If a variable cannot be controlled, record the variable
- New approaches to collection of samples/research
  - Nitrocellulose blots to collect samples of DNA/RNA/protein without affecting tumor samples
  - Blots of tissue on microscope slide
  - Smaller aliquots to support novel methods of research
  - Expanded use of paraffin blocks
  - Tissue microarrays
    - A statistical sample of paraffin blocks
    - Design to include controls

17.5 Issues in Processing of Specimens

- Processing of specimens should be rapid and matched to the specimens
  - Personnel should be flexible in their training so they can perform multiple tasks
  - The quality assurance program should cover processing
  - SOPs should be developed for each step of processing
    - SOPs for each bodily fluid (e.g., serum, urine)
    - SOPs matched to banking of solid tissue
    - SOPs matched to preparation of each solid tissue/bodily fluid (e.g., paraffin block)
    - SOPs matched to investigator needs for prospective collection of solid tissue/bodily fluids
  - Times of processing should be documented in informatics system
    - Monitor and document parameters of fixation and tissue processing
    - Reversing effects of fixation and tissue processing
  - Sample aliquots should be obtained for quality control (QC)
  - Samples should be uniquely labeled
  - Processing of specimens should be matched to investigator needs
- Importance of correct labeling
  - Errors may occur in reading label
  - Labels may separate from the specimen
  - Bar coding (a method to limit problems with specimen identification)
    - Unique to smallest aliquot and recognizes any child samples

  - May be linked to extensive information in the associated informatics system
- Age, race, sex, history, prior therapy, outcome
- Specimen characteristics – size, times of collection and processing
- Storage information and sites on each aliquot
  - Personnel errors may occur in identifying specimens

17.6 Long-Term Storage of Biospecimens

- Options from liquid nitrogen to room temperature
- To maintain cell viability over time usually requires at least storage in liquid nitrogen vapor and no freeze-thaw cycles
- For frozen tissues, we know that −20 °C (non-self-defrost) is probably okay for 1 month, but after several months, tissues begin to lose molecular features compared to storage at −80 °C
- Differences between −80 °C and liquid nitrogen vapor storage are not obvious at the protein level, and in a study by the CHTN −80 °C storage was better at mRNA level

17.7 Informatics and Records of Collecting, Processing, Storage, and Distribution of Tissue Specimens

- There are many variables in collection, processing, storage, and distribution of specimens which cannot be accurately controlled
- Information on each aliquot and the associated variables are linked by a code which frequently is in the form of a bar code
- When variables cannot be controlled exactly, information as to times and conditions of processing to distribution should be documented in the informatics system
- Location at which each unique aliquot is stored is also included in the informatics system
- Detailed information is a critical component of the quality assurance program as is the quality control diagnosis
- When specimens are removed from storage, information should be documented as to their distribution or destruction
- Sometimes specimens may be removed from storage and further aliquoted
  - These new aliquots (child samples) should be uniquely labeled and identified in informatics system
- The information permits reconstruction of the history of the aliquot

17.8 Types of Specimens Provided by a Tissue Repository

- Tissue repositories can provide a narrow and/or wide range of specimens of tissue
- Many banks focus on the collection of samples of blood/blood products
- Other tissue repositories may provide only paraffin blocks or paraffin sections of tissue
- Many prospective or combined models of repositories can provide fresh specimens, frozen specimens, blood products, paraffin material, tissue microarrays, mRNA, microRNA, and/or DNA

17.9 Distribution of Specimens to Investigators

- Internally, investigators should arrange for specimen pickup at tissue repository
- External distribution should rely on reliable commercial couriers who permit and understand the transfer of biological specimens (e.g., Federal Express)
- Air shipments must meet the standards of the International Air Transport Association (IATA)
  - This includes training of at least one employee involved in shipping as to IATA requirements
  - Meeting IATA requirements is the responsibility of the biorepository, not the courier

- Shipments scheduled to avoid problems and identification of problems (e.g., Monday to Thursday)
  - Special arrangements should be made for shipments on Friday or Saturday
- Problems with shipments should be identified rapidly by investigators (postcard) and corrected
- The informatics system should track the distribution of specimens

17.10 Quality Assurance Program

- Quality assurance (QA) is the overall process by which the uniformity and the quality of all operations of an organization are optimized with an emphasis on quality
- QC is an aspect of QA that ensures that a process or a product meets defined standards
- Good manufacturing practices should be followed as described by the International Standardization Organization (ISO) (ISO9001)
- Standard operating procedures (SOPs) are standardized approaches to performance of specific operations of an organization (e.g., collecting serum from patients with idiopathic pulmonary hypertension)
- SOPs are written detailed descriptions of each activity performed by a tissue repository so that the activity could be performed reliably by personnel who have not performed the activity previously
  - SOPs are reviewed annually and revised if necessary
  - A change in an SOP is made by authorized designated supervisors, and the change is dated as to when made and identified as to who made the change
  - Replaced SOPs maintained as archival SOPs so prior activity can be understood and reconstructed
  - SOPs organized into a procedure manual
- Equipment standardization, maintenance, and operations are an integral component of QA
- Audits are written periodic evaluations of operating procedures and infrastructure of the tissue repository
  - Conducted regularly by QA employees
  - Adherence of all employees to the QA program
  - Submitted to upper management and chief executive officer of the tissue repository
  - Examples of audits are provided in Box 17.2
- Audits evaluate all aspects of the QA program
- QA personnel and administrative issues
  - Personnel of the QA program should be independent of management of operations, sales, etc.
  - Head of QA reports to head of the organization or designates
  - Audits reported to head of the organization and problems with operations or QC should be corrected
  - QA personnel should aid with SOPs
- Surveys document user satisfaction
  - Short term – how specimens are distributed/shipped (Is dry ice adequate? Did it arrive on time?)
  - Long term – satisfaction of investigators/users with interactions with the biorepository and quantity, quality, and usefulness of specimens
  - Reviewed by QA personnel
- Quality control of solid tissues – quality control monitors the process and the usefulness of the tissue specimens provided to investigators
  - Verification of diagnosis of actual specimens provided to investigators or stored in tissue banks

Box 17.2. Audits that may be incorporated into a QA program

- Adherence to SOPs
- Storage of specimens
- Equipment repair and maintenance
- Equipment monitoring (e.g., freezer temperatures)
- Current training of personnel
- Quality of specimens (QC)
- Survey of investigators as to their satisfaction with products and problems with products

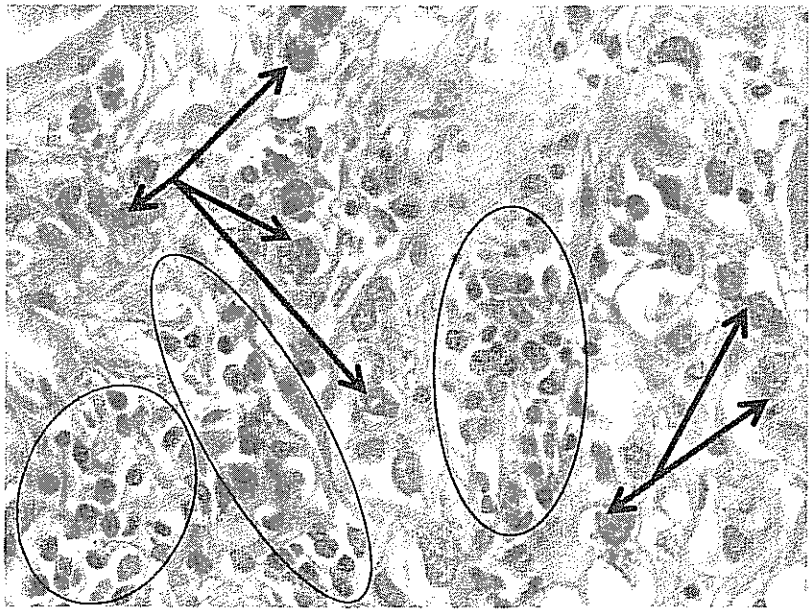

Fig. 17.1 This figure demonstrates a breast cancer (H&E, X630) in which the cancer cells, examples demonstrated by the *black arrows*, are extensively intermixed with inflammatory cells (within *ovals*). The cancer cells could be removed and analyzed as a separate cellular compartment by laser capture microdissection, a very labor intensive process. Enrichment of the number of cancer cells by micro-/macrodissection in which a paraffin or frozen section of the tumor is used as a guide to remove noncancer cells cannot be accomplished due to the close intermixture of cancer and noncancer cells. Of note, this tumor contains more inflammatory cells than cancer cells (about a 3:1 mixture), and many of the inflammatory cells are plasma cells. If the tumor were analyzed by homogenization and extraction of mRNA, microRNA, DNA, and/or protein, most of the extracted molecules would come primarily from noncancer cells. This critical limitation on the use of such specimens in molecular research is important for investigators to understand

- Diagnosis confirmed by mirror image aliquots examined by a pathologist. This is a critical component of QC
- For tumors, identify % tumor and of the tumor % necrosis, % fibrosis/mucin and % tumor nuclei
- Additional quality control can be added which can include molecular quality control (e.g., RIN number via Agilent 2100 system or actual molecular analysis with RT-Q-PCR)
- For some very infiltrative tumors such as pancreas or prostate or when there is extensive inflammation, microdissection or macrodissection may be necessary to isolate neoplastic cells
  - This can be done on frozen or paraffin embedded tissues
  - Approaches for investigators should be documented and there should be cost recovery (Figs. 17.1 and 17.2)
- Quality control in the collection of biological fluids
  - Fluids collected include fluids from fine needle aspiration, whole blood, serum, plasma, buffy coats, urine, saliva, and rare fluids such as pancreatic duct fluid ("juice") and cerebrospinal fluid
- Diagnosis is based upon overall diagnosis of the patient or sometimes a subdiagnosis (e.g., pancreatic cancer in patient with diabetes)
- Follow SOPs for collection, processing, storage, and distribution
- Speed of processing and storage after obtaining the specimens is important

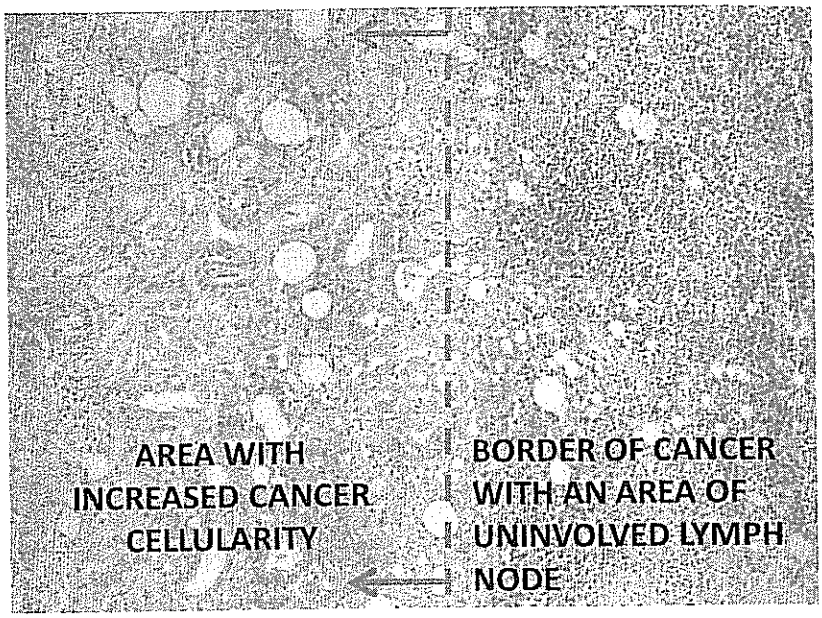

Fig. 17.2 This figure demonstrates a pancreatic adenocarcinoma partially involving a lymph node. To analyze the molecular expression of genes specifically in the cells of the pancreatic cancer, the contribution of noncancer cells (i.e., in this case, the lymphocytes and other cells of the uninvolved area of the lymph node) must be excluded. Micro-/macrodissection of the specimen to only include the area with increased tumor cellularity can be performed by removing the border area before cutting sections from the paraffin block. Such paraffin sections would only include the area indicated by the *dashed line* and *blue arrows*

- Try to complete processing and storage of blood/products, urine, and saliva within 4 h
  - Clinical followup after 6 months may be necessary to ensure a correct diagnosis

17.11 Education and Training in Issues Related to Tissue Repositories

- To produce uniform, standard specimens and to minimize bias, personnel must be carefully trained in SOPs and overall repository operations
- Potential sources of specimens and issues of repository science
- Records of training must be maintained
- To protect patients, all personnel must be trained in ethical issues (IRB) and privacy issues (HIPAA requirements)
- As discussed subsequently, all personnel must be trained in safety
- Just as important as educating repository personnel is the education of investigators using the repository
  - It is important for tissue repository pathologists to ensure investigators understand the tissues they are using in their research
  - This includes the potential for specimens to cause bias, optimization of tissues to support their research, and alternative tissues
  - Also, the investigator and all their personnel must be trained in biohazards

17.12 Matching Available Tissues to Investigator Needs for Tissues

- Pathologist needs to review each investigator's request for tissues and if appropriate, discuss the request with the investigator as well as alternative tissues

  - For example, a request for "smooth muscle" or "fat" is too general in that these tissues vary biologically depending on their source
- For example, smooth muscle from the uterus responds to estrogens and has active estrogen receptors
- In contrast, smooth muscle from arteries responds to hormones that control blood pressure
  - Some other tissues may be similar enough that additional sources can be identified
  - Sources of some tissues may be primarily met from autopsies or tissues removed for transplant
- Some tissue requests are very difficult to meet and this must be explained to investigators
  - Large numbers of rare diseases (e.g., synovial cell sarcomas)
  - Specimens with multiple requirements (male breast cancer from males less than 40 years of age who are African-Americans)
- The addition of each requirement makes the specimens more and more difficult to identify
- For example, African-Americans represent 30% of the clinical population at UAB, and breast cancers in males are uncommon and even rarer would be a breast cancer in a young male
  - Specimens that are atypical for the disease
- For example, papillary serous carcinomas of the ovary in African-Americans – a tumor that is infrequent in African-Americans
  - Specimens of primary tumors of large size in heavily screened populations in which large tumors are uncommon
  - Resection specimens processed very rapidly (e.g., 5 min)
    - Meeting this requirement would disrupt normal clinical operations
  - Determine whether or not there is scientific evidence for the need for difficult to meet requests
  - Requests for failed donor tissues or tissues removed at transplant (e.g., lungs from patients with cystic fibrosis)

17.13 Equitable Distribution of Tissues

- Tissue repositories should attempt to equitably distribute tissues to investigators
- Typically investigators are assigned priorities for specific specimens, but if specimens are not in high demand, typically specimens are assigned on a rotating basis
- Some requests are too labor intensive to be met (e.g., obtaining a whole spinal cord)
- Some requests cannot be met because of diagnostic requirements (e.g., samples of fresh ductal carcinoma in situ [DCIS] of the breast); DCIS is used totally in diagnosis to exclude microinvasion
- Some requests are so restrictive that very few specimens would meet the request

17.14 Annotation of Tissue Specimens

- Some basic information is needed on any tissue aliquot being studied including most importantly its correct diagnosis
  - Age, race, and sex are important information that usually can be readily obtained
- The need for more extensive annotation usually depends upon the use of the tissues
- Other than clinical outcome, more extensive annotation usually is project specific, so general approaches to annotation may not be adequate
- Some information needed for annotation may not be available in charts; thus, for some studies and most epidemiology studies, the extent of specific annotation may need to be designed with some of the data obtained directly from patients (e.g., family histories, environmental exposures)
  - In such cases, the study would have to provide extra resources to facilitate such annotation
- In many cases, annotation of specifics may not be needed. In these cases, it may be more efficient to collect the annotation after the specimens and matching annotations are requested

17.15 Bias Affecting Usefulness of Tissues

- Bias results from differences in cases and controls unrelated to the process or diseases being studied
- Bias causes an incorrect interpretation that experimental differences between cases and controls are due to the disease; instead, the differences are due to variables in the way the tissues are obtained and/or experiment is performed
- Bias cannot be detected by routine statistical analysis
- Bias typically is detected when there is failure to confirm a previous result using a different independent population
- The chance that bias will be important in a study increases with the number of variables being studied
- Bias may be introduced into research projects due to samples of tissue when there are differences between cases and controls due to varying conditions in collecting, processing, storing, or distributing tissues as well as differences in the populations from which samples are obtained
- Use of SOPs aid in reducing the chance of bias
- Specific specimen factors causing differences between samples and controls due to bias. These include the issues in Box 17.3
- Specific population differences: examples that may cause experimental differences in case versus controls due to populations are listed in Box 17.4
- Avoiding bias: the chance of bias can be minimized by ensuring cases and controls match (e.g., # of diabetics in cases is equivalent to # of diabetics in controls)

Box 17.3. Potential causes of bias Due to tissues

- Number of freeze-thaw cycles
- Procedures for collection, processing, and storage
- Collection sites
- Type of specimens (e.g., EDTA plasma vs. citrate plasma)
- Differences in specimen containers
- Failure to use SOPs or SOPs that vary between cases and controls
- Site-specific bias (unidentified)

Box 17.4. Potential causes of bias due to population

- Demographics (e.g., age, race, sex)
- Comorbid conditions (e.g., diabetes)
- Donor stress
- Homeostasis (fed vs. fasting)
- Diurnal variations (time when sample is drawn)

Box 17.5. Issues to be considered in an MTA

- Transfer to third parties
- Use of specimens
- Indemnification
- Commercial uses
- Intellectual property

17.16 Administrative Issues

- The transfer of tissues or tissue products to extramural investigators usually should require a material transfer agreement (MTA) between the institutions/organizations sending and receiving the specimens
- An MTA is unidirectional and addresses issues, for example, in transferring tissues from a collecting site to a site where the investigator is located
- MTAs actually should facilitate the transfer of specimens among institutions
  - Examples of issues that should be addressed in MTAs are listed in Box 17.5

17.17 Shipping

- Transfer of specimens outside the organization collecting, processing, storing, and/or distributing tissue specimens
- Use of reliable courier which is equipped to ship human specimens
- International shipping is very difficult and is problematic unless specimens are shipped by specialized couriers; international shipments are very expensive
- Even though assigned to a courier, the shipping site is responsible for meeting shipping requirements, specifically the requirements of the IATA
  - Someone at the shipping site must be trained in IATA requirements
- Shipping of flammables (e.g., specimens in alcohols) is limited in quantity by IATA

17.18 Regulatory and Ethical Issues Affecting Tissue Repositories

- Ethical issues – the IRB
  - IRB approval should be obtained for tissue biorepository; all IRB approvals, exemptions, and decisions as to a classification of "nonhuman research" are local
    - Collection of remnant tissues not needed for diagnosis
  - Specimens in the biorepository are identified as to the identification of donor in the repository – full review by IRB may be required as well as informed consent from the source of the tissue
  - All specimens are anonymized – could be classified by IRB as "nonhuman research" or IRB may require an expedited review
- The need for informed consent could be waived
  - IRB approval of investigators using a tissue repository
    - If specimens are provided as anonymized, research may be classified by the IRB as "nonhuman research." IRB approval may be necessary
    - If specimens are de-identified, research may be classified by the IRB as "nonhuman research" or IRB approval may be required
    - If specimens are identified, research will usually require IRB review and approval
    - For any situation, review by the IRB is necessary to determine how the work of the biorepositories is classified

Box 17.6. List of HIPAA 18 identifiers

- Names
- All geographical subdivisions smaller than a State, including complete zip codes (see HIPAA discussion on website) (http://www.hhs.gov/ocr/privacy/hipaa/understanding/summary/index.html)
- All elements of dates (except year) for dates directly related to an individual (see HIPAA description on website), for ages over 89, the elements may be aggregated into a single category of age 90 or older
- Phone numbers
- Fax numbers
- Electronic mail addresses
- Social Security numbers
- Medical records numbers
- Health plan beneficiary numbers
- Account numbers
- Certificate/license numbers
- Vehicle identifiers and serial numbers, including license plate numbers
- Device identifiers and serial numbers
- Web Universal Resource Locators (URLs)
- Internet Protocol (IP) address numbers
- Biometric identifiers, including finger and voice prints
- Full face photographic images and any comparable images
- Any other unique identifying number, characteristic, or code (note this does not mean the unique code assigned by the investigator to code the data)

- Privacy issues – HIPAA
  - HIPAA specifies 18 HIPAA-designated identifiers (Box 17.6)
- If all these 18 identifiers are excluded from the identification of the tissue specimen, HIPAA requirements do not apply to these tissue specimens
  - Some of the 18 HIPAA identifiers are dates (e.g., birth date)
- Age can be substituted for birth date in most research
  - If dates are required, a limited dataset can be constructed which excludes other HIPAA identifiers except dates
- If a limited dataset is used by an investigator and the investigator signs a data use agreement with the source of the clinical information or tissue, HIPAA also does not apply
- Informed consent
  - May be waived by local IRB for de-identified and/or anonymized specimens
  - There is no perfect time or place to obtain informed consent
    - At clinic – space and time limited
    - In the operating room – area, space, and time limited, patient is under stress and is fasting
    - After surgery (patient may require pain medication)
    - Trying to obtain consent after discharge is usually unsuccessful
  - Specimens obtained specifically for research (e.g., extra solid tissue, bodily fluids) usually require informed consent from patients
  - Informed consent from patients frequently requires significant resources because tissue repositories have no direct relationship with patients
  - Obtaining informed consent from some patients to use tissues in biomedical research may be a problem due to religious, social, or ethnic considerations/views
- Return of research results to patients/repositories
  - Research results may be wrong or their interpretation may be incorrect
  - Research results may not be applicable to all subpopulations
  - Research results returned to patients or potentially involved in medical decisions in the USA must be from CLIA-approved laboratories
  - Significant liability may occur if research results cause harm to patients
  - Research results may be accidentally released
  - Return of research results requires significant infrastructure and is expensive

17.19 Cost Recovery

- It is illegal in many countries to sell human tissues; however, recovery of costs associated with collection, processing, storage, and distribution of tissues is appropriate. Such cost recovery may be necessary to permit continued operation of a human tissue biorepository
- Some form of cost recovery aids in ensuring appropriate respect for and efficient utilization of human tissues
- Cost recovery is an appropriate component of long-term financial stability of tissue repositories
- The extent of cost recovery is usually based upon resources supporting a biorepository

17.20 Safety in Tissue Repositories

- A tissue repository has many potential dangers, especially biohazards and chemical hazards
  - Potential biohazards include blood-borne pathogens (e.g., HIV, hepatitis B), tuberculosis, antibiotic resistant bacteria, and prions
  - All tissues, even paraffin blocks, must be handled with universal precautions
  - Chemical hazards include formaldehyde, alcohols, and xylene
  - Physical hazards include slippery wet and paraffin coated floors, as well as dangers to employees from other individuals
  - If volatile reagents (e.g., xylene) are used or stored, fire is a potential danger

  - Electrical hazards are represented by improperly grounded equipment as well as personal appliances (e.g., radios)
- Safety program – the safety program of a tissue repository may be partially independent or a part of the safety program of the umbrella organization
  - A safety program should have a safety committee and a safety officer
  - A safety plan is developed to minimize the chance of injury including the use of engineering practices (e.g., good ventilation and drains), which includes the use of safety equipment (e.g., safety glasses, hoods)
  - The safety plan includes review of the potential dangers of each employee/visitor based upon not only the work performed but also the area of work
  - A safety training program is established for personnel that include special training focused on blood-borne pathogens, chemical hazards, and if appropriate, formaldehyde
- The safety program applies to personnel working in the work area where such chemicals are present or stored; updated yearly
  - The safety plan is reviewed yearly and when incidents occur, the safety plan is modified to prevent their recurrence
  - Vaccinations for hepatitis B should be offered to all employees who are in contact with fresh or frozen human tissues
  - Because repositories provide specimens outside their organization, it is a good idea to require that outside personnel receiving these specimens be educated in biohazards including universal precautions
  - To protect against chemical hazards, material safety data sheets (MSDS) should be available to all employees using chemicals to which the MSDS apply

17.21 Informatics

- A biorepository should have an informatics program if the repository is large enough to warrant it. The informatics program should save the repository effort and money. This might include
  - A bar coding system
  - An investigator component to keep track of the details of all investigator requests
  - A donor component to record details of the donor (e.g., age, race, sex, history, clinical information such as prior therapy)
  - The donor component might be combined with a specimen component to follow the collection, processing, storing, and distributing of each unique aliquot usually via a unique bar code
  - Unique common data elements to avoid redundancy should be used
- These should typically utilize a pathology vocabulary as well as more general designations (e.g., any breast cancer)
  - Required fields should be minimized, and the navigation should follow the business plan of the repository (e.g., the order of how specimens are collected and processed)
  - If specimens are identified as to the identity of tissue sources, the system must meet HIPAA security requirements including installing the system on a secure server behind a firewall, the use of strong passwords, and the maintenance of audit trails for even viewing of identified patient information
  - It may be beneficial to divide the database into an "identified" component which is small and has very limited access and a de-identified database devoid of all 18 HIPAA identifiers with most of the donor data; this minimizes "read only" audit trails
  - In order to meet NCI and ISBER Best Practices, the informatics system should keep track of the history of specimens including the times and details of tissue processing (e.g., freeze-thaw cycles)

17.22 Challenges in Meeting Specimens for Genomic Studies

- Many tissue banks have SOPs that are not designed to meet the stringent demands of current genomic studies (e.g., 0.15 g of

a tumor with ≥80% tumor nuclear cellularity and ≤30% necrosis); see Fig. 17.1
- Most tumors, except for selected types, cannot meet this standard because such a requirement is not characteristic of the growth pattern of the tumor and supplying such specimens would require costly microdissection (see Fig. 17.2)
- Also, these and similar requirements will introduce bias into studies in that the results will only apply to a very select group of tumors and not to tumors in general
- The costs with such collections exceed the funds provided for collecting, processing, and microdissection of such cases
- Specimens for genomic studies are best collected prospectively; however, the costs of detailed prospective collections (e.g., matching samples of blood) may be over $1,000 per case (patient) due to the many collected specimens which do not meet requirements

17.23 Challenges Leading to New Directions in Tissue Repositories

- All these challenges require relatively novel approaches and significant additional resources
- Problems
  - Neoadjuvant therapy – identify cases treated with neoadjuvant therapy
- They may or may not be useful for specific research projects
  - Smaller sizes of tumor – some tumors are too small to obtain samples
  - If only in situ lesions are present, some specimens must be submitted totally to exclude microinvasion
  - Biopsies of metastatic lesions may be so small that they must be submitted totally
  - Tissues from metastatic lesions may be unavailable because imaging is used in lieu of biopsy of suspected lesions
- Potential solutions
  - Nitrocellulose blots extract RNA, DNA, and proteins from tissues
- Proteins can be extracted from nitrocellulose blots and used for discovery; new approaches using housekeeping proteins for quantitating specific proteins may be developed for blots
- mRNA and microRNA can be extracted from nitrocellulose and together with housekeeping genes can determine mRNA and microRNA levels using RT-Q-PCR
- DNA can be extracted from nitrocellulose blot and analyzed
  - Blots of tumors on glass microscopic slides can be used to analyze proteins, DNA and RNA
  - For small tumors, in situ lesions, or metastatic lesions, increase the use of diagnostic paraffin blocks and construct tissue microarrays
  - For all small lesions including fine needle aspirates, try and obtain extra samples for consented patients
  - Consider using tissues from autopsies of patients with metastatic disease; perform such autopsies rapidly (warm autopsy) to make tissues more useful
  - Use of micro-/macrodissection and laser capture microdissection if enriched (Fig. 17.2) or pure (Fig. 17.1) cellular populations are needed

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Title Page

Use of Human Specimens in Research: The Evolving US Regulatory, Policy, and Scientific Landscape

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Abstract

The use of human specimens in research has contributed to significant scientific and medical advancements. However, the development of sophisticated whole genome and informatics technologies and the increase in specimen and data sharing have raised new questions about the identifiability of specimens and the protection of participants in human specimen research.

In the US, new regulations and policies are being considered to address these changes. This paper discusses the current and proposed regulations as they apply to specimen research, as well as relevant policy discussions. It summarizes the ways that researchers and other stakeholders can provide their input to these discussions and policy development efforts. Input from all the stakeholders in specimen research will be essential for the development of policies that facilitate such research while at the same time protecting the rights and welfare of research participants.

Keywords: human specimen research, human subjects protection regulations, research policy, ethical issues, biorepositories, personalized medicine.

Use of Human Specimens in Research: The Evolving US Regulatory, Policy, and Scientific Landscape

Human specimens and human specimen biorepositories play a key role in scientific and medical advancement, and will continue to play a critical role in the future, particularly towards efforts to develop individualized medicine and targeted therapies. However, their widespread use raises a number of challenges, particularly those related to the current regulatory, policy and scientific landscape. Discussed in this paper is the potential importance of human specimens and human specimen biorepositories to research and the current and evolving regulatory, policy and scientific landscape in the US as it applies to such research.

Introduction:

Importance of Human Specimens in Research. The use of human specimens in biomedical research has been critical to the development of current medical care. Although animal cell lines and specimens are useful in most research, molecular features of animal specimens frequently are different from those of matching human

specimens just as animal diseases are, in general, different from human diseases. For example, it was recently reported that mice are a poor model for studying the genetics of human inflammatory disease.[1,2] The importance of archival clinical specimens in support of biomedical advances has been elegantly described by Korn as the "intellectual foundation of modern medicine".[3] Indeed, since this publication, significant additional advances in medical care have been based on research using human specimens. Included are many major advances that have not only changed therapies of diseases, but also have resulted in new concepts of human biology.

Therapeutic advances and approaches to medical care now are beginning to target unique molecular features of pathways that have been identified in human specimens as important to the development of specific diseases. The potential uses of molecular targeting have resulted in approaches to treat the unique features of an individual patient's disease (e.g., personalized or individualized medical care). These approaches will require even greater use of human specimens both in medical research and to aid in clinical decisions.[4,5,6,7]

Specific targeting of features of molecular pathways has resulted in novel molecular directed therapies for once untreatable diseases, especially targeting specific molecules in different types of cancer. For example, the cellular surface receptor HER-2, was initially identified using archival human tissues and reported to be important in causing an aggressive subtype of breast cancer. Studies on human tissue led to the development of approaches to therapy that specifically target the HER-2 receptor.[8] Thus, a once poor prognostic feature of one type of breast cancer was changed by an effective strategy of molecular targeting to a good prognostic feature.[9,10,11] In addition, HER-2 targeted therapies are now being expanded to treat other forms of cancer, such as gastric cancer.[12]

Recent advances using human tissue have expanded and changed our concepts of human cellular biology resulting in potential new approaches to the treatment of a wide variety of diseases. For example, it was discovered that some messenger RNAs (mRNAs), which are the precursor molecules that permit the production of all proteins, were regulated in human and other cells by a newly identified category of small molecule, microRNA. [13] Up regulation or down regulation of specific proteins

may be involved in the development, progression, or severity of specific diseases; the level of these proteins frequently are affected by microRNAs.[13,14] Significantly, microRNAs are potential targets for specific therapies and the production of these proteins may be modulated by targeted microRNAs.[15,16] Several other paradigm-changing discoveries based on the use of human tissues also have altered our concepts of normal biology as well as causes of disease.[5,17] Given the demonstrated importance of human specimens to biomedical research and their role in developing approaches to targeted medicine, demand for human specimens has increased dramatically over the past several decades.[18] A number of types of human specimen biorepositories have been developed to help meet this demand.

Surgical Specimen Biorepositories. Because only a small proportion of tissues removed surgically are required for diagnosis, remnant tissues can be used to support biomedical research by constructing additional paraffin blocks for research or providing the remnant tissues as frozen and/or fresh viable tissue. The tissue required for diagnosis is embedded in paraffin and in addition to providing

diagnostic information, also can be used in future research. Other innovative approaches to obtain samples for research may be applied to specimens that are too small or are in situ lesions; these include obtaining nitrocellulose blots as tissue aliquots.[19] These aliquots of residual surgical specimens may be stored for future research in various types of biorepositories.

The Cooperative Human Tissue Network (CHTN) is a prospective biorepository model in which tissues are collected specifically to meet investigator requests.[20] Alternatively, tissues can be banked for future use in research biorepositories following a standard operating procedure (SOP); such a banking model is typically utilized by Specialized Programs in Research Excellence (SPORE)[21] and the National Cancer Institute Clinical Cooperative Group Banks.[22] Each of these two models, prospective and banking, has advantages and disadvantages. The prospective model has the advantage of providing specimens which exactly meet an investigator's needs and is a model in which generally all specimens are utilized; however, neither specimens nor clinical outcomes are immediately available from prospective

biorepositories because both must be collected over time. The banking model typically has multiple specimens immediately available as well as clinical outcomes for the specimens provided. The banking model is most appropriate for cases in which it is necessary to collect clinical or longitudinal data and the participants are being followed over time. The disadvantages to the banking model are that the SOPs that are used in collecting and processing specimens may not meet investigator needs and requirements, especially for use with future technologies that may not yet be developed, and that many specimens may never be used. The underutilization of specimens in biobanks has recently been proposed as an important ethical issue.[23] While both types of biorepositories are useful models, careful attention is needed to the design of the biorepository to ensure that specimens are optimally utilized. [6,17]

While human specimens and human specimen biorepositories continue to be important to scientific and medical advances, the availability of large numbers of specimens and extensive associated demographic and clinical data, the powerful

new genetic and genomic technologies such as whole genome sequencing and the enormous and rapid advances in informatics, raise a number of evolving ethical, legal and social issues related to the use of human specimens. Discussed below are the current US regulations and policies as they relate to specimen research, including human specimen biorepositories, and the evolving scientific and policy landscape in the US.

The Current US Regulatory, Policy and Scientific Landscape

Federal Regulations That May Apply to Human Specimen Research. In the US, there are three important federal regulations that may apply to the use of human tissue and associated data in research, the "Common Rule," promulgated by the Department of Health and Human Services (HHS) and codified at Code of Federal Regulations (CFR) title 45 part 46, Subpart A[24], the US Food and Drug Administration (FDA) human subjects regulations at 21 CFR part 50[25], 56[26], and 812[27]and the Health Insurance Portability and Accountability Act (HIPAA) Privacy

Rule (45 CFR part 160 and Subparts A and E of part 164)[28] and Security Rule (45 CFR part 160 and Subparts A and C of part 164).[28] Each of these regulations is discussed in further detail below.

The Common Rule: The Common Rule has been codified by 15 US federal departments and agencies and applies to all research involving human subjects that is "conducted, supported or otherwise subject to regulation by any federal department or agency which takes appropriate administrative action to make this policy applicable to such research." Each of these 15 federal department or agencies has a codification of the Common Rule which is equivalent to 45 CFR 46, Subpart A[1]. The Rule includes requirements for Institutional Review Board (IRB) review and informed consent for human subjects research.

The Common Rule defines "research" as a systematic investigation, including research development, testing and evaluation, designed to develop or contribute to

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[1] Although they have not issued the Common Rule in regulations, three other agencies have signed onto the Rule. For a complete list of agencies that follow the Common Rule, see http://www.hhs.gov/ohrp/humansubjects/commonrule/.

generalizable knowledge. Sometimes the definition is challenging to interpret as the difference between "research" and uses of patient specimens and associated data in education or clinically relevant activities becomes blurred.[29] Therefore, researchers and pathologists should consult their local IRB for guidance before beginning activities involving human specimens.

The Common Rule defines a human subject as a living individual about whom an investigator conducting research obtains either data through intervention or interaction with the individual; or identifiable private information [45 CFR 46.102(f)]. Therefore, the Common Rule would apply when specimens or associated information are obtained for research from a living individual through intervention or interaction with the individual, such as a blood draw or cheek swab, or when residual specimens taken during the course of routine care are collected prospectively for research purposes. It would also apply when identifiable specimens are used for research (i.e., when the identity of the subject is or may readily be ascertained by the investigator or associated with the specimens).

Furthermore, in order for research involving humans specimens to be considered human subjects research under the Common Rule, the individuals must be living. Thus, according to the Common Rule, as currently written, research involving material from deceased individuals (e.g. autopsy material) or the use of specimens that are completely anonymous (i.e. a link to subject identity does not exist), would not be subject to the Common Rule, although state and local regulations and policies may apply.

Under certain circumstances, research using coded specimens, that is, specimens for which identifying information has been replaced with a code, may not be considered human subjects research if certain conditions have been met[30]. The creation of a human specimen biorepository for research purposes is considered to be a research activity and would be considered to involve human subjects research if specimens and/or associated data are being collected through interaction or intervention with a living individual or if the human specimen repository includes the collection, distribution or use of identifiable private information.

For research using human specimens that is considered human subjects research, the Common Rule generally requires review by an Institutional Review Board (IRB) and informed consent from the subject/participant. However, research involving the collection or study of existing data, documents, records, pathological specimens, or diagnostic specimens may be exempt from these requirements if the information is recorded by the investigator in such a manner that subjects cannot be identified, directly or through identifiers linked to the subjects. [45 CFR 46.101(b)(4)]. Furthermore, the requirement for informed consent for use of human specimens may be waived by the IRB when all of the following conditions are met [45 CFR 46.116(d)]:

- The research involves no more than minimal risk to the subjects;
- The waiver or alteration will not adversely affect the rights and welfare of the subjects;
- The research could not practicably be carried out without the waiver or alteration; and

- Whenever appropriate, the subjects will be provided with additional pertinent information after participation.[2]

It is important to note that these requirements are based on the Common Rule as currently written. As discussed later in this paper, changes to these requirements are under consideration.[31]

The FDA Human Subjects Regulations: The second set of significant US federal regulations that may apply to the collection and use of human specimens for research are the Food and Drug Administration (FDA) regulations, 21 CFR 50[25], 56[26], and 812[27]. The FDA regulations are similar, but not identical, to the Common Rule. The FDA regulations apply to all clinical investigations regulated by the FDA under sections 505(i) and 520(g) of the Federal Food, Drug, and Cosmetic Act, as well as clinical investigations that support applications for research or marketing permits for products regulated by the FDA. Among other products included within

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[2] This requirement was intended to apply to 'deception research,' and generally believed to rarely, if ever, apply to research on human specimens.

the scope are drugs for human use, medical devices for human use and biological products for human use. 21 CFR 50 covers informed consent requirements and 21 CFR 56 covers IRB review requirements. 21 CFR 812.2(a) applies to all clinical investigations of devices to determine safety and effectiveness unless the device investigation is exempt under 812.2(c).

The FDA regulations define a human subject differently than the Common Rule. The FDA regulations at 21 CRF 50.3(g) and 56.102(e) define a human subject as an individual who is or becomes a participant in research, either as a recipient of the test article or as a control. A subject may be either a healthy human or a patient. (See 21 CFR 50.3(g) and 56.102(e)). The device regulations define a subject as an individual on whom or on whose specimen an investigational device is used. (See 21 CFR part 812).

Unlike the Common Rule, the FDA regulations do not require the research participant/subject to be identifiable for the regulations to apply. Furthermore, unlike the Common Rule, the FDA exemptions to the requirement for informed consent are limited to emergency, life threatening situations, and military

operations. This may pose challenges for some studies involving human specimens (e.g. the development of assays using archived specimens when it is difficult or impossible to contact the individual to obtain informed consent). In order to address this issue, the FDA issued guidance stipulating that the FDA would exercise enforcement discretion with regard to requiring informed consent when leftover human specimens that are not individually identifiable are used in FDA-regulated in-vitro diagnostic investigations, if certain conditions specified in the guidance are met.[32]

The Health Insurance Portability and Accountability Act, Privacy and Security Rules: The third major set of US federal regulations that may be relevant to some human specimen research is the Health Insurance Portability and Accountability Act (HIPAA) Privacy and Security Rules.[28] The Privacy Rule regulates the uses and disclosures of individually identifiable health information by "covered entities" (health care providers, health plans, and health care clearinghouses). While the Privacy Rule does not apply to the use of human specimens per se, it may apply to uses and disclosures of the health information that may be associated with the

specimens. The Privacy Rule generally requires patient authorization for uses and disclosures of health information that is individually identifiable. [See 45 CFR part 164.508]. Authorization is a similar but not identical concept to informed consent. Informed consent is the process by which subjects are informed about the risks and benefits of participating in research whereas authorization is solely a permission to allow researchers to use or disclose defined protected health information.

The ways in which protected health information may be used and disclosed for research is summarized in Table 1. Patient authorization is not required if the information to be used or disclosed is de-identified according to the Privacy Rule's requirements at Section 164.514 (See Table 2) or a "Limited Data Set" (See Table 3) pursuant to a Data Use Agreement that meets the requirements of the Rule. Patient authorization for the uses and disclosures of protected health information is also not required if an IRB has waived the requirement for authorization according to criteria stipulated in the Rule, for purposes "preparatory to research" or for research solely on decedents if certain representations are made to the IRB. The US

Department of Health and Human Services has provided additional guidance on how the Privacy Rule applies to research[33], as well as guidance on de-identification.[34]

The HIPAA Security Rule establishes national standards to protect individuals' electronic personal health information that is created, received, used, or maintained by covered entities.[28] The Security Rule (45 CFR Part 160 and Subparts A and C of Part 164) includes requirements for appropriate administrative, physical and technical safeguards to ensure the confidentiality, integrity, and security of electronic protected health information. These requirements may apply to research databases, such as those that may be associated with specimen collections, including those of individual investigators.

Other Applicable Regulations and Policies: In addition to the aforementioned US federal regulations, there may be state and local regulations or funding agency policies that may apply to human specimen research. For example, some states have their own human subjects regulations (e.g. New York, Maryland, Virginia, and California).[35] In addition, state laws concerning genetic testing, genetic or medical record privacy also may apply and these may vary considerably from state to

state.[35] Certain human specimen research funded by the National Institutes of Health (NIH) may be subject to resource and data sharing policies, such as the NIH policy on genome-wide association studies. This policy calls for investigators funded by the NIH for genome-wide association studies to share de-identified genotypic and phenotypic data through a centralized NIH data repository.[36]

In other countries, there may be different ethical and privacy regulations and policies that may apply to the use of human specimens and associated data in research.[37]. These regulations and policies, especially in the European Union, are evolving rapidly and need to be considered when international collaborations are involved in research involving human specimens and/or data.

The Evolving Legal and Ethical Landscape Related to Human Specimen Research

Some of the aforementioned US regulations governing human subjects research were written a decade or two ago. Since that time, the research environment has

evolved dramatically from research conducted in single laboratories to national and international multi-site collaborations between academia, government, industry and non-profit entities. Specimen and data sharing has also increased significantly, with many funding agencies now expecting broad sharing of research tools and data. Furthermore, the advent of affordable whole genome technologies, the increase in research databases, and implementation of electronic health records have raised new questions about privacy. At the same time, advances in technology are raising new questions regarding the identifiability of specimens and genomic data.

A number of cases in the media, both in the US and abroad have underscored some of the ethical, legal and social issues related to the use of human specimens in research. The Alder Hey organs scandal in the UK, involved the unauthorized removal, retention, and disposal of human tissue, including children's organs, from 1988 to 1995, and led to the Human Tissue Act 2004, and the creation of the Human Tissue Authority.[38,39] A number of cases in the US, have also highlighted important ethical issues related to the use of human specimens for research. Issues related to informed consent and the commercial use of tissue were highlighted in a recent best

seller[40] concerning Henrietta Lacks, the daughter of a poor African- American tobacco farmer whose specimens were obtained without her knowledge or consent and used to develop cell lines which have been shared broadly and sold throughout the world. More recently, the posting of Henrietta Lacks' genomic sequence on a publicly available website without the consent of her family led to its removal.[41,42] The retention of blood spots obtained from newborn children for research without parental consent led to a lawsuit in Texas resulting in the destruction of approximately 5 million samples.[43,44] Other cases such as the Moore Case[45], Canavan Case[46], and Catalona Case[47, 48, 49]involved lawsuits regarding claims of private ownership of human specimens used in research. In none of these cases did courts find that research participants had any ownership rights to their tissue, although the courts noted the importance of informed consent.[50] To date, there is no federal law addressing the ownership of human tissue.

In another case that received attention in the US, specimens were collected from members of the Havasupai tribe for research on diabetes. The specimens were later

used for studies of migration and other purposes that the tribe found objectionable. Tribal members sued the investigators and the university claiming fraud, breach of fiduciary duty, intentional infliction of emotional distress, negligence, conversion and lack of informed consent. The lawsuit also alleged that the researchers allowed wholesale transfer of blood samples from laboratory to laboratory and university to university and that many samples could not be accounted for. The lawsuit was settled with a payment of $700,000 to the tribe along with the return of the blood samples , any derivatives, and associated data and documentation.[51,52]

These cases demonstrate the importance of informed consent and transparency in specimen research. They also demonstrate the need to respect cultural perspectives in the conduct of such research, and the importance of having systems in place for tracking specimens when they are distributed for additional research and mechanisms for ensuring specimens are used consistent with informed consent.

These cases also illustrate the need for sound governance mechanisms and best practices for the collection, storage, distribution and use of human specimens in

research. A number of best practices have been developed in this area. These include the International Society for Biological Biorepositories Best Practices[53], the National Cancer Institute (NCI) Best Practices[54], and the Organisation for Economic Cooperation and Development (OECD) Guidelines for Human Biobanks and Genetic Research Databases.[55]

In the US, new regulations and policies are being considered to address the changes in the research environment.[56] The changes in the regulations being contemplated were discussed in An Advanced Notice of Proposed Rulemaking (ANPRM) entitled "Enhancing Protections for Research Subjects and Reducing Burden, Delay, and Ambiguity for Investigators" published on July 26, 2011.[31] The ANPRM discusses a number of changes to the Common Rule that are being contemplated to provide additional protections for participants of research, as well as reforms to reduce the burden to the research community.

The ANPRM addressed a number of issues related to human specimen research and invited comments on them. Among the issues for which public comments were solicited is whether specimens should in themselves be considered identifiable,

whether consent should be required for unidentified specimens, and whether a broad (non-specific) consent for future use of tissues should be considered acceptable. In addition, a new category of research was discussed, an "excused" category of research involving secondary use of specimens and identifiable information in which consent is required but there is no IRB review, unless the researcher plans to contact subjects with individual research results. Additional issues related to the use of human specimens discussed in the ANPRM are summarized in Table 4. During the public comment period of 90 days, more than 1,100 comments were received in response to the proposed Rule.

A Notice of Proposed Rulemaking is only one, preliminary step in the regulation making process. The Administrative Procedure Act, Pub.L. 79-404, 60 Stat. 237, governs the way federal agencies may propose and establish regulations. This Act generally requires agencies to publish all proposed new regulations in the Federal Register at least 30 days before they take effect and provide a way for the public to comment on the proposed regulation. The agency can then decide whether to

proceed with the rulemaking process and if so, incorporate the public comments into a Notice of Proposed Rulemaking. This Notice of Proposed Rulemaking is issued for public comment before finalizing and publishing a Final Rule. However, an agency may decide to take no further action at any step of this process. At the time of the writing of this article, a Notice of Proposed Rulemaking on the proposed changes to the Common Rule has not been issued.

The scientific and policy landscape is evolving to reflect new ethical issues and privacy challenges related to advancements in science and technology. Recent studies demonstrating the potential to identify individuals by their genomic data, even when stripped of traditional identifiers has raised new questions about how best to protect participants who contribute their specimens to research. Homer and colleagues demonstrated that they could detect an individual's SNP profile in a mixture of DNA from 1,000 individuals.[57] This led to a change in NIH's data sharing policies for whole genome association studies to provide further protection of aggregate genome wide association study data shared through the Database of

Genonotypes and Phenotypes (dbGaP).[58,59] In another more recent study, researchers were able to identify anonymous DNA donors in the 1,000 Genomes Project by matching their DNA sequences to publicly available genealogy databases.[60]

Another area of considerable discussion related to human specimen research is when individual research results should be provided to research participants. The issue of when individual research results should be returned to research participants is not addressed explicitly in US federal regulations.

From an ethical perspective, the issue of return of results and incidental findings has been debated for many years, and a number of groups have made recommendations in this area.[61,62] Arguments for return of results include respect for persons, beneficence, reciprocity, justice, and the duty to rescue.

Arguments against return of research results include the view that the original intent is an altruistic donation to help research, that return of research results would promote a therapeutic misconception, and perhaps most importantly, that

harms can accrue when individual research findings that are incorrect or have not been validated are returned to participants or their physicians.

More recent discussions have focused on when and how research results and incidental findings should be returned to individuals from genomic biobanks.[63] However, the return of research results from genomic biobanks is complex, with not only ethical implications, but legal and practical implications; thus considerable caution in the return of such findings is needed.[64,65]

The issues raised by the advent of genomic technologies are being explored by the Presidential Commission for the Study of Bioethical Issues, a panel of experts who advises the President on bioethical issues arising from advances in biomedicine and related areas of science and technology. The Commission recently issued a report entitled, "Privacy and Progress in Whole Genome Sequencing".[66] The Commission recommended strong baseline protections for whole genome sequence data and urged federal and state governments to ensure a consistent floor of individual privacy protections covering whole genome sequence data across state lines. They also recommended that clinicians and researchers use robust and understandable

informed consent procedures when conducting whole genome sequencing and that the federal government facilitate broad public access to the important clinical advances that result from whole genome sequencing. As its next project, the Commission has taken up the return of incidental findings, including those arising during the course of genomic research and other research on human specimens. While the Commission's recommendations do not constitute official policy guidance, these discussions and other policy development efforts are likely to have a significant impact on biorepositories and the use of human specimens in research and should be followed closely by the research community.

Summary and Conclusions

As the scientific and policy landscape continues to evolve in the US, it will be important for researchers and other stakeholders to provide input as new regulations and policies are developed. Researchers, research participants, and

other relevant stakeholders can follow publication of regulations and policies in the Federal Register[67] and Regulations.gov[68] and provide comments through Regulations.gov during the public comment period. Additionally, the International Society for Biological and Environmental Repositories (ISBER)[69] Science Policy Committee tracks policy and regulatory developments in the US and abroad, disseminates information to ISBER members and provides comments on behalf of ISBER. Nonetheless, comments from individuals as well as groups representing them are also important. Active engagement of all the relevant stakeholders will be essential to help inform the development of policies related to the use of specimens in research that will allow important research to proceed, while at the same time protecting participants of such research, their privacy and the confidentiality of their data. Responsible stewardship of specimens used for research will be critical to ensure that public trust is maintained in the research enterprise.

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Conflicts of Interest

M.J.B. is a member of the International Society for Biological and Environmental Repositories (ISBER). W.E.G. operates tumor banks as part of the Breast, Pancreatic and Cervical SPOREs at the University of Alabama at Birmingham and the Pulmonary Hypertension Break- through Initiative and prospective tissue repositories as part of the Cooperative Human Tissue Network and the Comprehensive Cancer Center and is a member of ISBER. He is also a member of the ethics group of the U54 grant, Morehouse School of Medicine/Tuskegee University/University of Alabama at Birmingham Comprehensive Cancer Center Partnership.

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Table 1.

Ways in Which Protected Health Information Can be Used and Disclosed by Covered Entities For Research Under the HIPAA Privacy Rule [45 CFR parts 160, 162, and 164]:

- With individual's authorization for research [See 45 CFR part 164.508]
- Without individual's authorization if one of the following applies and other conditions in the Rule have been met [See 45 CFR part 164.512]:
    - IRB or Privacy Board waiver
    - Preparatory to research (with certain representations)
    - Limited data set (with data use agreement)
    - De-identified dataset
    - Research solely on decedents
    - Informed consent, waiver of informed consent, or permission before compliance date

Table 2. De-Identified Data Set[12]

The Privacy Rule allows a covered entity to de-identify data by removing all 18 elements that could be used to identify the individual or the individual's relatives, employers, or household members; these elements are enumerated in the Privacy Rule. The covered entity also must have no actual knowledge that the remaining information could be used alone or in combination with other information to identify the individual who is the subject of the information. Under this method, the identifiers that must be removed are the following:

1. Names.
2. All geographic subdivisions smaller than a state, including street address, city, county, precinct, ZIP Code, and their equivalent geographical codes, except for the initial three digits of a ZIP Code if, according to the current publicly available data from the Bureau of the Census:
    a. The geographic unit formed by combining all ZIP Codes with the same three initial digits contains more than 20,000 people.
4. Telephone numbers.
5. Facsimile numbers.
6. Electronic mail addresses.
7. Social security numbers.
8. Medical record numbers.
9. Health plan beneficiary numbers.
10. Account numbers.
11. Certificate/license numbers.
12. Vehicle identifiers and serial numbers, including license plate numbers.
13. Device identifiers and serial numbers.
14. Web universal resource locators (URLs).

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[1] Excerpt taken from "Protecting Personal Health Information in Research: Understanding the HIPAA Privacy Rule". U.S. Department of Health and Human Services.

[2] Note Covered Entities may also use statistical methods to establish de-identification instead of removing all 18 identifiers, [See 45 CFR part 164.514(b)].

b. The initial three digits of a ZIP Code for all such geographic units containing 20,000 or fewer people are changed to 000.

3. All elements of dates (except year) for dates directly related to an individual, including birth date, admission date, discharge date, date of death; and all ages over 89 and all elements of dates (including year) indicative of such age, except that such ages and elements may be aggregated into a single category of age 90 or older.

15. Internet protocol (IP) address numbers.
16. Biometric identifiers, including fingerprints and voiceprints.
17. Full-face photographic images and any comparable images.
18. Any other unique identifying number, characteristic, or code, unless otherwise permitted by the Privacy Rule for re-identification.

Table 3

Limited Dataset Under the Privacy Rule[1]

A limited data set is described as health information that excludes certain, listed direct identifiers (see below) but that may include city; state; ZIP Code; elements of date; and other numbers, characteristics, or codes not listed as direct identifiers. The direct identifiers listed in the Privacy Rule's limited data set provisions apply both to information about the individual and to information about the individual's relatives, employers, or household members. The following identifiers must be removed from health information if the data are to qualify as a limited data set:

1. Names.
2. Postal address information, other than town or city, state, and ZIP Code.
3. Telephone numbers.
4. Fax numbers.
5. Electronic mail addresses.
6. Social security numbers.
7. Medical record numbers.
8. Health plan beneficiary numbers.
9. Account numbers.
10. Certificate/license numbers.
11. Vehicle identifiers and serial numbers, including license plate numbers.
12. Device identifiers and serial numbers.
13. Web universal resource locators (URLs).
14. Internet protocol (IP) address numbers.
15. Biometric identifiers, including fingerprints and voiceprints.
16. Full-face photographic images and any comparable images.

[1] Excerpt taken from "Protecting Personal Health Information in Research: Understanding the HIPAA Privacy Rule". U.S. Department of Health and Human Services.

Table 4. Specimen-Related Issues on Which Comments Were Solicited in the Advanced Notice of Proposed Rulemaking, "Human subjects research protections: enhancing protections for research subjects and reducing burden, delay, and ambiguity for investigators"[1].

- Specified data security protections calibrated to the level of identifiability
- Identifiability of specimens
- Consent requirements for research use of specimens stripped of identifiers
- Acceptability of broad consent for specimen research
- Whether there should be an "excused" category of research involving secondary use of specimens and identifiable information in which consent is required but there is no IRB review, unless PI plans to contact subjects with individual research results

[1] Federal Register. July 26, 2011. 76 CFR 44512. HHS-OPHS-2011-005.

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Exosomes: A novel pathway of local and distant intercellular communication that facilitates the growth and metastasis of neoplastic lesions

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Running Head: Exosomes and Cancer

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ABSTRACT:

Normal and diseased cells release bilayered membrane bound nanovesicles into interstitial spaces and into bodily fluids. A subgroup of such microvesicles are called exosomes and are described in blood as 30-100nm in diameter, spherical to cup-shaped nanoparticles with specific surface molecular characteristics (e.g., expression of the tetraspanins CD9, CD81 and CD63). Extracellular microvesicles provide local signals (e.g., autocrine and paracrine) as well as distant endocrine signals to cells via the transfer of their contents which include signal proteins, lipids, microRNAs and functional mRNAs. Exosomes and related microvesicles also aid cells in exporting less ~~un~~needed molecules as well as potentially harmful molecules including drugs; in the case of neoplasia, the export of chemotherapeutic drugs may facilitate cellular chemoresistance.

Cancers have adapted the exosome and related microvesicles as a pathway by which neoplastic cells communicate with each other (autocrine) as well as with non-neoplastic cells (paracrine and endocrine); via this pathway, cancers suppress the immune system and establish fertile local and distant environments to support neoplastic growth, invasion, and metastases. Because exosomes mirror and bind to the cells from which they arise, they can be used for delivery of drugs, vaccines and gene therapy, as biomarkers and as targets. This manuscript reviews how exosomes and related extracellular microvesicles facilitate the progression and metastases of cancers and describes how these microvesicles may impact clinical care.

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Keywords: exosomes, microvesicles, immune surveillance, microenvironment, autocrine, paracrine, endocrine, mRNA, microRNA, early detection, prognosis, risk, delivery vehicle

AN OVERVIEW OF EXOSOMES AND RELATED MICROVESICLES IN CANCER:

Exosomes are a subcategory of bilayer membrane-bound nanovesicles released from normal and diseased cells into interstitial spaces and in some cases, into bodily fluids. Exosomes also are released by cultured cells into media. Exosomes are defined and separated from other vesicles based upon their source, method of isolation, sizes and surface markers. Exosomes and other vesicles frequently are unrecognized as to their importance to physiological and pathological processes because they are essentially invisible; their small sizes keep them suspended in fluids so that their effects may not be identified in that their contents and functions would seem to be consistent with those of soluble molecules. Exosomes and related microvesicles were once thought to be artifacts and/or cellular trash, but now exosomes are accepted as a component of a newly identified intercellular communication system that can modulate the functions of target cells. The involvement of exosomes and related microvesicles in providing autocrine (i.e., local signals between the same cell type, e.g., cancer cells), paracrine (local signals between different cell types such as between epithelial cancer cells and stromal cells) and endocrine signals (i.e. distant signals between any types of ~~-~~cells usually carried ~~via~~ in bodily fluids [blood]) ~~via their molecular contents~~ has led to the frequent use of the term, signalosomes, being applied to these structures.

A specific subtype of exosome/vesicle/particle is released by tumor cells (tumor-derived exosomes or TD-exosomes). TD-exosomes/microvesicles may be released into the interstitial space or even directly into lymphatics or into pseudo-capillaries formed by tumors. Also, the neovascularity of malignant tumors is thought to be especially leaky and its permeability may

be influenced by exosomes, cytokines and other local molecules such as vascular endothelial growth factor (VEGF).

The presence of TD-exosomes in patients with malignant tumors leads to several important issues and concepts (1-23).

- Exosomes/microvesicles in the blood of patients with tumors are composed of both exosomes/vesicles from normal cells, diseased cells (comorbid conditions), as well as TD-exosomes/microvesicles (Table 1). TD-exosomes are characteristic of ~~the~~ tumor cells and have different molecular characteristics than microvesicles from other sources.

- Tumor cells may release more microvesicles than other cells and TD-exosomes may have easier access to the vascular system and thus may be selectively increased in blood compared to microvesicles from other sources.

- Smaller microvesicles with specific molecular surface characteristics may selectively reach the blood and larger microvesicles may remain in the interstitial space and selectively provide autocrine and paracrine signals to stromal, inflammatory and endothelial cells.

- Once specific cellular populations within the tumor are affected by TD/exosomes/microvesicles, various modulatory loops may be established which facilitate the growth, progression, and cellular dissemination of the tumor.

- Via autocrine interactions, TD-exosomes may stimulate malignant cells to grow, move and invade the vascular-lymphatic system and disseminate via chemotaxis to nodal and

other metastatic sites. Exosomes may establish favorable environments at potential metastatic sites and may aid the survival of neoplastic cells at sites of metastases.

- As part of establishing specific micro-environments and interacting with various cellular compartments, chemotaxis is likely to be a very important though poorly described signaling pathway of exosomes.

- Some tumors frequently induce thrombosis and hence may cause an increase in microvesicles/microparticles that are released from platelets. These microvesicles have to be experimentally separated from microvesicles derived from actual malignant cells or other types of cells.

- TD-exosomes can interact with the normal cells of the immune system to reduce immune surveillance by increasing cells which inhibit immunity, decreasing antigen presenting cells and inhibiting T and NK cells.

- Exosomes function in removal of waste products, drugs, and/or less~~other~~ ~~un~~necessary molecules from cells and hence exosomes may facilitate chemoresistance.

- Exosomes/microvesicles have been reported to transfer oncogenic features among malignant cells as well as transiently to non-neoplastic cells, suggesting the probability of lateral transfer of phenotypic features among cells.

- Immunoseparations suggest that distinct populations of exosomes may have different molecular characteristics and may arise from different intracellular areas of polarized cells.

- Exosomes may be useful clinically and in translational research by improving the analysis of biomarkers, delivery of therapeutic and preventive drugs, vaccines, gene therapy and as targets to improve immunity.

CHARACTERISTICS OF EXOSOMES:

Signaling by exosomes and microvesicles is likely to be affected by their morphologic and molecular features. In gaining entrance to the lymphatic-vascular system, smaller nanoparticles are likely to have an advantage because the microvesicles must pass through spaces between endothelial cells via mechanisms which are not adequately defined. Similarly, the molecular characteristics of the external surfaces of exosomes may affect this passage and some exosomes are taken up by endothelial cells and influence "secondary exosomes" released from endothelial cells.

Subgroup of Microvesicles: Exosomes typically can be collected from blood via a series of steps of centrifugation to remove larger cellular debris and sometimes by filtration through a 100-220 nm filter to exclude larger microvesicles including apoptotic blebs. Subsequently, exosomes are pelleted by ultra-centrifugation at about 100,000 g for 60 minutes, washed and suspended by ultracentrifugation in a sucrose gradient. After these steps, exosomes isolated from blood typically are described via electron microscopy as spherical to cup-shaped, bi-layered, membrane bound nanovesicles ranging in size from 30 to 100 nm in greatest dimension and by Western blotting of the exosomal preparations as demonstrating the presence of multiple molecular features, including specific tetraspanins (1-12).

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When the "usual" steps are not taken during isolation of exosomes, the nanoparticles presented in preparations frequently are described as "microvesicles", exosome-like particles, microparticles or sometimes oncosomes if the microvesicles transmit oncogenic properties to target cells. Currently, there is confusion because results based on "microvesicles" are sometimes reported as from "exosomes" and vice versa. Both the sources of microvesicles and their isolation affect their characteristics; a 220 nm filter or smaller will limit the sizes of microvesicles, sucrose gradient separations will affect sizes via their density and ~~will~~ may remove molecules absorbed on exosomes.

If specific microvesicles are involved in intercellular communication, is it important to classify microvesicles based on their sizes and shapes? The effects of size-shape characteristics have not been described adequately, but size, shape and surface characteristics may affect how exosomes gain access to bodily fluids and deliver signals to ~~the~~ specific cells with which they interact (12).

Molecules phenotypically expressed on the external surface of microvesicles separate nanovesicles originating from multivesicular bodies (MVB) from those arising from budding from cellular membranes, and hence, define different subgroups of microvesicles that may have different morphological and molecular characteristics, different sources and perhaps, different functions. Those markers identified by western blotting may not be present on all microvesicles and may not be associated with the microvesicles producing specific functional characteristics.

Thus, the sizes, shapes and surface molecules of microvesicles may affect their concentration in blood (12). Microvesicles isolated from fluids other than blood may have different morphological features; microvesicles isolated from semen are described as being up to 500 mm in size and containing intra-vesicular structures, from spent culture media as biphasic in size (200-400 nm and 600 to 1000 nm), and from saliva as doughnuts with a height to width ratio of 0.04 (23-25). Exosomes isolated from ascites are described as being similar to exosomes isolated from matching blood (26).

The release of exosomes from normal and diseased (i.e., non-neoplastic) cells is affected by multiple factors including calcium, calcium ionophores, PI3 kinase (PI3K), heat, ischemia, cellular stresses, pH, phorbol esters, and loss of cellular attachment (13,27-33). Hypoxia increases specific exosomes which, in turn, may stimulate angiogenesis (13). As would be expected, factors that affect the formation of MVBs, from which exosomes specifically arise, affect the secretion of exosomes, so exosomes are decreased by inhibitors of PI3K (wortmannin).

Multiple molecular factors control exosomes including the small GTPases (Rab) and p53 via TSAP6 (34-36).The complex of syndecan 1-syntenin and ALIX affects the intraluminal budding of exosomal membranes and together with endosomal sorting complex responsible for transport (ESCRT-III), these molecules cleave the membrane buds. This complex may also be involved in growth factor transport (37,38) and may be related to control in exosomes of selective protein sorting by ceramide (39). In addition, heparanase, which may aid in the formation of the syndecan-1-syntenin-ALIX complex, also stimulates the secretion of classic exosomes (40).

Although these molecular features affect the secretion of exosomes, our knowledge of the overall process is rudimentary. This same pathway may also be utilized by specific viruses in their release from cells (33,36).

Kinetics of Exosomes: The kinetics of release of exosomes is relatively rapid. After loading of MCF-7 cells with doxorubicin, exosomes containing doxorubicin were present at cellular membranes within 3 hours (23). Similarly, after stimulation of mast cells with alloantigens, exosomes were released within 30 minutes (41). The internalization of exosomes by target cells also is a rapid, active process; one-third of dendritic cells (DCs) imported exosomes at 37°C within 2 hours. Internalization requires metabolic energy and is decreased by EDTA, cytochalasin D, and incubation of the target cells at 4°C (31,41,42).

Molecular Characteristics of Exosomes: Exosomes are defined by specific molecular features including the phenotypic expression of specific surface molecules. The commonly used molecular markers of exosomes are the surface tetraspanins, CD9, CD63, CD81, CD82 and CD151 and other molecules including ICAM-1, $\alpha_v\beta_3$ (CD51, CD61), integrin, ALIX, externalized phosphatidylserine, milk fat globule-E8/lactoferrin (MFG-E8), CD80, CD86, CD96, Rab-5b and MHC class I and MHC class II complexes (7-9,31,32,41-46). Tetraspanins may form a net incorporating different tetraspanins and neighboring molecules and this net may be incorporated in multivesicular bodies (MVBs) and into exosomes (47). Exosomes/microvesicles may vary with their origin; the transferrin receptor is in exosomes from reticulocytes and CD11a and CD54 in exosomes from hematopoietic cells. Exosomes from tumors may express molecular features of the tumor type (e.g., melanin A from melanomas [5,10-12,17-19,31,33,46,47]).

Recent studies using colorectal cells have suggested that when cells are polarized in 3-D cultures, two subtypes of exosomes are secreted from different intracellular locations. Specifically, approaches were used via which larger microvesicles are excluded from preparations, i.e. , filtration through a 100 nm filter and concentration of the specimen through a 5 kD molecular weight limit membrane. Exosomes were then separated from this preparation by immunoaffinity based on a colonic epithelial cell type specific molecule, A33, followed by immunoaffinity separation based on Ep-CAM. The two types of exosomes that were identified had slightly over 50% of proteins in common; however, there were about 20% different proteins in the A33 cell specific fraction and about 25% different proteins in the Ep-CAM fraction. The Ep-CAM fraction seemed to be primarily enriched in apical proteins, e.g., CD44 and tetraspanins, while the A33 fraction was enriched in basolateral proteins including clathrin and MHC class I molecules. This observation, if independently confirmed in other cell types, will greatly expand our understanding of exosomes. Similarly, it is likely that there are other subtypes of exosomes, e.g., an exosomes-population involved specifically in cellular waste management.

In that exosomes not only act in intercellular communication, but also in exporting waste and less unneeded products from cells (48,49), exosomes containing waste products are likely to have surface molecules which instruct phagocytes to remove them (48). For example, the phenotypic surface expression of galectin-5 has been proposed as being important in the removal of reticulocyte exosomes by macrophages ~~(~~ (50). Other signals may be analogous to those by which apoptotic cells are phagocytosed.

The release of exosomes from normal and diseased cells provides signals to other cells via their contents which include a wide range of functional molecules including hundreds of different proteins, mRNAs, microRNAs and lipids (16,25,51-55). When mRNA contained in murine exosomes from mast cells reacted with human mast cells, murine proteins were produced in the human cells (524).

Although microRNAs are a relatively new area of investigation, their impact on human diseases has been demonstrated to be important (56,57—). Thus, the observations that exosomes from multiple types of tumors contain microRNAs is ~~both~~ noteworthy but~~and~~ controversial, in that some reports indicate that most circulating microRNAs are outside exosomes. Of interest, the controversy is not whether exosomes contain microRNAs, but rather the proportion of circulating microRNAs that are within exosomes (58,59). It would likely be advantageous for all circulating RNAs to be within exosomes. Exosomes are a vehicle that not only can protect RNAs, but potentially can deliver RNAs to specific target cells in which microRNAs may modulate mRNAs (56). Thus, the exosome is likely to be both a typical chaperone for some microRNAs as well as their other molecular contents as well as a specific delivery pathway. As previously indicated, exosomes are invisible unless they are specifically looked-for. To support the concept of invisibility, a study challenging the reports indicating that most circulating microRNAs are outside exosomes reported that most circulating microRNAs from serum and saliva are contained ~~in~~ within exosomes (60—). ~~The controversy is not whether microRNAs contain microRNAs, but rather the proportion of circulating microRNAs that are in exosomes.~~ These

studies have used serum and plasma from normal patients; t~~T~~his issue may be more important in ~~related to~~exosomes released from tumors from which exosomes are released than to exosomes released from non-neoplastic cells. Tumor cells have a high rate of cellular death and when the cells of tumors die, their surviving contents, including RNA, including microRNA~~s~~'s-argonaute 2~~A~~ cojmplexes would be dumped into the interstitial space and can be picked up by the lymphatic-vascular system~~( )~~. Thus, depending upon the rate of death of the malignant cells of specific tumors, it would not be surprising to find microRNA both inside and outside exosomes, especially if complexed with argonaute 2~~Ag~~. Similarly, as discussed, the method of isolation of exosomes may strongly impact the RNAs in tumor-derived exosomes. Specifically, a proportion of exosomes may be destroyed on isolation, releasing their contents into solution (58). The resolution of the question of whether circulation microRNAs are primarily within or outside exosomes will obviously require much more study. The issue may well relate to function in that~~. As suggested,~~ microRNAs contained in exosomes may be more biologically active with respect to specific uptake by targeted cells.

Exosomes separated from saliva can affect proteins in oral keratinocytes of humans (25); thus, not only do exosomes act as an endocrine system, but also provide autocrine, paracrine and other signals to target cells. For example, microvesicles can be exchanged among touching cells using tunneling nanotube networks (5~~4,5~~5,61). Although molecules in exosomes can provide molecular signals to modulate target cells, how target cells accept or reject the hundreds of different molecules contained in microvesicles that could provide different signals is not

understood. More is known about the molecular signals that are accepted by target cells than those that are ignored.

How exosomes from different cells target specific cells also is yet to be clarified; however, there must be selective interactions of exosomes with target cells, probably via interactions with molecules on the external surface of exosomes with interacting surface molecules on target cells. Exosomes bind to the breast carcinoma cell line, BT-549, via annexins A2 and A6 and the molecules of lipid rafts (32,62~~56~~). The active uptake of exosomes by DCs can be only partially blocked by monoclonal antibodies to surface molecules including CD9, CD11a, CD54, CD81 and $\alpha v\beta_3$ integrin. Each monoclonal antibody blocked internalization of exosomes by only about 30% of control values, but monoclonal antibody to MFG-E8, a surface molecule that potentiates uptake of apoptotic cells by macrophages, stimulated the uptake of exosomes by about 30% (42). How combinations of surface signals potentiate uptake of exosomes remains to be clarified. Of interest, tetraspanins are involved in the fusion of HIV-1 with T cells and facilitate uptake of HIV-1 by dendritic cells and macrophages; thus understanding the cellular uptake of exosomes might be increased by a better characterization of how cells are infected with viral particles and apoptotic cells are phagocytosized by macrophages (47,63~~57~~).

How do cells decide which molecules to package into exosomes? Waste or less needed molecules could be packaged into one type of vesicle, but signal molecules into different microvesicles. Alternatively, if both types of molecules are packaged into the same microvesicles, are the ~~waste~~waste -molecules destined for degradation ~~modified molecularly~~

via molecular modification (e.g., ubiquinated) so that they are ignored by the target cells. The JAB1/CSN5, a component of COP9, seems to play a role in the sorting of proteins that are ubiquitinated into exosomes and this might be a step in processing of waste molecules (64,65~~58,59~~). Other waste molecules may be sorted via the ESCRT-0,-I and –II pathways. The tetrapanins, especially CD81, complex with some viral particles, e.g., hepatitis C (HCV), to promote their incorporation into exosomes (63~~57~~); however, viral particles are much more complex than single molecules. Also, ceramide and heparanase seem to pay a role in transferring potential signal molecules into exosomes (39,40).

Functions of Exosomes in Normal Physiology: Exosomes have been isolated from most normal biological fluids including bronchoalveolar fluid, cerebrospinal fluid, blood, urine, saliva, breast milk, amniotic fluids, semen and synovial fluid as well as diseased fluids including ascites and pleural effusions. (24-26,51,66-69~~0,60-62~~). Thus, exosomes are likely to play important roles in normal physiology as well as in diseases. Most roles in normal physiology have not been well characterized except with regard to the functions of exosomes in the immune system.

The Actions of Exosomes in the Normal Immune System: Exosomes play important roles in the intercellular communication among normal immune cells, but communication may typically be dysregulated in disease processes. Most immune cells have been reported to secrete exosomes. During their normal functions, mast cells, dendritic cells (DCs), immature antigen presenting cells (imAPCs) macrophages, T and B lymphocytes and NK cells use exosomes to communicate (reviewed in 10-12).

An excellent example of how exosomes affect the immune system is their involvement in maternal fetal tolerance (~~63-67~~70-76). To protect the fetus, the placenta releases exosomes, some of which phenotypically express Fas ligand (FasL), an immunosuppressive as well as an anti-inflammatory molecule (~~68-70~~77-79). In addition, syncytiotrophoblasts secrete exosomes which express ULBP1 to 5 which are ligands to the NKG2D receptor expressed on γδT, NK and CD8+ T cells. During pregnancy, an interaction of the NKG2D receptor with its ligands decreases the functions of immune cells (~~65~~72) as well as the microRNA cluster "chromosome 19 microRNA cluster (C19MC)" which suppresses immunity and facilitates the growth of some tumors (75,76). Thus, exosomes are involved in the decreased immunity that is necessary for successful pregnancies.

Exosomes are an important feature in the processing of antigens by antigen presenting cells (APCs). Dendritic cells (DCs) can utilize exosomes in the presentation of processed antigens to NK cells and T lymphocytes. In this process, stimulating antigens or antigen-peptides are combined in MHC class II complexes present on the surfaces of some exosomes and these MHC class II antigen complexes then stimulate immune cells. The presentation of antigen/peptides by exosomes is much more efficient than if exosomes are not involved (~~71-74~~80-83). At least two different subpopulations of DCs are required for optimal stimulation of T cells by exosomes. DCs carrying an MHC class II antigen complex secrete exosomes which carry the complex; t~~. T~~hese exosomes can be taken up by $CD8^{-}\alpha$, $CD80^{+}$, and $CD86^{+}$ DCs which can then process the exosomes so that an exosome-HLA class II antigen-peptide complex can be presented by DCs to immune cells such as $CD4^{+}$ T cells. The activation of $CD4^{+}$ T cells can be

amplified via transferring the HLA class II antigen-peptide complex to multiple DCs independent of their expression of HLA class II complexes (~~71,72~~80,81).

$CD8\alpha^+$ DCs control peripheral immune tolerance via modulation of the responses of $CD4^+$ T cells to alloantigens (42). Processing of exosomes by mature DCs is required for stimulation of MHC class I restricted $CD8^+$ T cell responses. Adjuvant molecules such as ligands for Toll-like receptors 3 and 9 aid in this stimulation (~~74~~83). In addition, mature B cells may be required for optimally stimulating T cells by exosomes released by DCs (84)~~75)~~.

Other effects of exosomes on immune cells have been reported; specifically mast cells secrete exosomes that activate B and T lymphocytes and DCs. Exosomes released *in vivo* from murine mast cells facilitated the maturation of DCs and increased the ability of DCs to present antigens to T lymphocytes. The heat shock proteins, HSP60 and HSP70, from exosomes have been reported to be very important for the presentation of antigens by dendritic cells (41). Exosome-like particles isolated from the spleen can modify $CD4^+$, $CD25^-$ T cells and convert them into $CD4^+$, $CD25^+$, $Foxp3^+$ T regulatory cells (Tregs). Tregs act to inhibit autoreactive T cells and thus, suppress dysregulated immune responses and minimize autoimmunity (85~~76~~). Exosomes also are sometimes involved in dysregulation of normal immunity and affect autoimmunity (~~77~~86). Depending upon the source cells and their molecular content, exosomes may suppress or atypically stimulate pathways of the immune system suggesting an involvement of exosomes in autoimmunity.~~.~~ Exosomes from synovial fibroblasts collected from patients with rheumatoid arthritis, but not osteoarthritis contain a membrane form of TNFα which potentiates the signaling of NF-κB and suppresses apoptosis of activated T cells. This may increase the severity

of rheumatoid arthritis (87)~~HG Zhang)~~. Alternatively, exosomes from genetically modified immunosuppressive immune dendritic cells (e.g., bone marrow derived dendritic cells (BMDCs) can reduce autoimmunity. BMD(s) expressing interleukin 10 (IC-10) or exposed to IL-10 release exosomes that inhibit both the inflammation and arthritis caused by injections of collagen into mice (~~Kim et al.).~~88). Similarly, when BMDCs are modified to express TGF-β1, the development of inflammatory bowel disease in mice secondary to dextran sodium sulfate is inhibited by exosomes from the TGF-β1 genetically modified BMDCs (89~~Cai~~).

Normal functions of exosomes in the export of waste products, ~~unnecessary~~ less needed molecules and harmful molecules: Cells use exosomes to export waste products, molecules no longer as useful as cells differentiate and molecules such as drugs that may be harmful to cells. This pathway can eliminate proteins without signal sequences and permit their secretion. Exosomes are especially important during cellular differentiation when large amounts of molecules must be removed from differentiating cells in order to optimize their more differentiated functions, e.g., metabolic active hematopoietic precursor cells which differentiate to anucleate relatively inactive red blood cells containing primarily hemoglobin (5048).

Exosomes and neoplasia: Exosomes are secreted by most types of cells including those of neoplastic lesions. The microvesicles released from probably all malignant cells are designated as tumor derived (TD). TD-microvesicles have been studied primarily in bodily fluids (e.g., blood) and media from cultured cells.

Exosomes/microvesicles in bodily fluids are complex as is the molecular composition of bodily fluids because both microvesicles and free molecules in bodily fluids are from normal and diseased cells. Similarly, as noted previously, exosomes and microvesicles as well as their contents and effects are invisible unless analyzed as a separate component of fluids. Thus, measurements of the molecular contents of TD-exosomes potentially should be just as sensitive and specific for clinical uses and in translational research as measurements of the same molecules external to exosomes. TD-exosomes/microvesicles mirror the molecular features of the source neoplastic lesions. For example, TD-exosomes in blood from patients with glioblastoma multiforme and high grade gliomas contain neural markers, e.g., LI-NCAM/CD171 (~~78-81~~88-91) and exosomes from melanomas that express molecules involved in melamine synthesis and other melanoma markers (e.g., Melan A/Mart 1 (5,46,94).

Exosomes decrease the immune surveillance of tumors: Via its normal functions, the immune system should minimize the development and progression of cancers; however, malignant lesions have been found to inhibit immune surveillance resulting in their more rapid growth, progression and dissemination (10-12,~~82-88~~95-101). Specifically, tumors have increased growth *in vivo* after injection of exosomes isolated from matching neoplastic lesions (95~~82~~). Exosomes ultimately were found to inhibit immunity by several mechanisms including increases in suppressive immune cells, decreases in the proliferation and cytotoxicity of NK and T cells and decreases in the number and functions of APCs (reviewed in 10-12). Thus, the decrease in immunity caused by neoplastic lesions is primarily mediated by exosomes released from tumors (~~82-89~~95-103).

The intercellular signals provided by TD-exosomes to the immune system probably vary with different types of cancers. Oral and ovarian epithelial neoplastic lesions secrete exosomes/microvesicles that contain Fas ligand (FasL), Trail or related molecules (e.g., TNFα) which can cause apoptosis of activated T lymphocytes via suppression of CD3ζ and Jak-3 (~~68-70,84~~77-79,97); however, some TD-exosomes which increase the growth of their source tumors do not contain Trail nor Fas ligand, but interact with NK cells to decrease the secretion of perforin, inhibit Jak-3 and decrease cyclin D3 (95~~82~~). Thus, TD-exosomes are likely to decrease immunity via several mechanisms. For example, exosomes containing TGFβ1 may decrease the expression of the NKG2D ~~receptor~~ receptor via in part, microRNA-1245, and reduce the activation of NK and $CD8^+$T cells (~~85~~98,99).

TD-exosomes also decrease immunity by decreasing the numbers and/or activity of APCs including dendritic cells. Signals from TD-exosomes increase the phosphorylation of Stat 3 and the expression of IL-6 in DCs decreasing the activity of DCs and the numbers of APCs via inhibiting the differentiation of $CD14^+$ monocytes into mature APCs (100~~88~~). In addition, $CD14^+$ cells shift to immunosuppressive $CD14^+$ HLA-$DR^{-/low}$ cells which release TGFβ to inhibit T cells (101,102~~86,87~~).

TD-exosomes increase myeloid-derived suppressor cells (MDSCs). In humans, the numbers of MDSCs in neoplastic lesions are correlated with the neoplastic progression and decreased patient survival, probably via inhibiting NK cells and $CD4^+$ and $CD8^+$ lymphocytes by MDSCs (103-105~~91~~). MDSCs ($CD11b^+$, Gr-1^+) are increased in neoplastic lesions, spleens and blood of mice with transplanted syngenic tumors and they are decreased in the blood and spleens of

mice by removal of transplanted tumors (~~90~~104). Exosomes increase MDSCs via TGFβ which increases prostaglandin E2 (~~92~~106). TD-exosomes also increase MDSCs via MyD88, but effects of TLR pathways on MDSCs should be considered carefully because of potential phenotypic changes in cells induced by long term cultures (9-11,~~93,94~~107,108). In addition to the actions of exosomes, release of cellular soluble mediators such as GM-CSF from neoplastic cells also may increase MDSCs.

Another immune suppressive cellular population that is increased by exosomes is T regulatory cells (Treg). TD-exosomes can induce the differentiation of $CD4^+$, $CD25^+$ T cells to $CD4^+$, $CD25^+$, $Foxp3^+$ Tregs via the phosphorylation of both Stat 3 and SMAD2/3 (100~~95~~).

Figure 1 summarizes how neoplastic lesions can partially avoid immune surveillance by secreting TD-exosomes which inhibit the activity of T and NK cells, by decreasing APCs, and by increasing cells which suppress immune surveillance such as Tregs, MDSCs, and $CD14^+$-HLA-$DR^{-/low}$ cells.

Non-immune effects of exosomes on primary malignant lesions: The development, progression and dissemination of malignant tumors depends upon a balance of cellular proliferation and cellular death (e.g., apoptosis, autophagy). TD-microvesicles can provide autocrine, paracrine, endocrine and other signals which can generate fertile environments to support malignant lesions and hence stimulate their growth, progression, and metastasis (Figure 2) (13-22).

An important pathway by which TD-exosomes modulate the local environment of cancers are those which stimulate angiogenesis and hence, increase the supply of oxygen and nutrients to the growing tumor (13,16-18,1,22,~~78-81~~90-93). Specifically, exosomes secreted by melanomas

are reported to facilitate the formation of spheroids by endothelial cells as well as budding of these spheroids. Also, cytokines, including TGFβ and VEGF, which stimulate angiogenesis are contained in exosomes secreted from malignant cells in hypoxic and similar environments (13,16). Similarly, the blood from patients with glioblastomas as well as from the media of their short term cultures have been reported to contain microvesicles with the pro-angiogenic proteins, angiogenin, VEGF, IL-6 and IL-8. The microvesicles were enriched in angiogenic proteins compared to the source glioblastomas and these microvesicles stimulated increases in the length of endothelial tubes similarly to angiogenic proteins (~~78-81~~90-93). Also, upon exposure to hypoxia, A431 cells secrete exosomes that contain molecules which stimulate angiogenesis and facilitate metastasis (13).

TD-exosomes may modulate the local growth of neoplastic lesions via autocrine signals provided via exosomes. Autocrine effects of exosomes have been reported to vary with cellular characteristics as well as cell type. For example, exosomes from pancreatic cancers increased apoptosis via the Notch Pathway if the cell lines were well differentiated, but not if the cell lines were poorly differentiated; thus, exosomal autocrine signals might inhibit the growth via stimulating apoptosis of some pancreatic neoplastic lesions, but not others (~~96,97~~110,111). Alternatively, autocrine signals mediated by exosomes from the BT-474 breast cancer cell line, gastric cancer cell lines, and glioma cells increased cellular proliferation (2,90,112~~98~~). The mechanism of the increased proliferation of the gastric cancer cell lines was thought to be via increased phosphorylation of Akt and ERK which with other downstream molecules are associated with cellular proliferation. Growth of the glioblastoma cell line, U87, was increased via exosomes derived from primary glioblastomas; while this growth was assumed to be

secondary to increased proliferation, decreased apoptosis was not excluded (90~~78~~). Of note, survivin as well as heat shock proteins, HSP70 and HSP90, may be increased in exosomes in association with cellular stresses. These proteins may inhibit apoptosis as well as increase cellular proliferation and invasion so they provide a strong stimulus to the microenvironment that can facilitate the growth and dissemination of primary tumors (31,~~99~~113).

In addition to exosomes, microvesicles of other types may be secreted by neoplastic cells into the local microenvironment of primary tumors. Specifically, PC-3, a prostatic cancer cell line, released~~s~~ microvesicles distinct from exosomes which stimulated fibroblasts~~. Following this stimulation,~~ and the fibroblasts responded by an increased release of microvesicles that stimulated PC-3 cells to migrate and invade. This paracrine loop associated with the CX3CL1 and CX3CR1 was not observed in LNCaP cells which are not as aggressive as PC-3 cells (14). The variable responses of PC-3 and LNCaP cells to microvesicles from fibroblasts might be due to different molecular features within or on exosomal surfaces such as the binding complex, type III receptor betaglycan and its associated ligand TGF-β which are expressed strongly in PC-3 cells, but not on or in LNCaP cells (15).

TD-exosomes can induce changes in fibroblasts indicative of a transition to myofibroblasts. Fibroblasts/myofibroblasts then may modulate the microenvironment (e.g. degradation of the extracellular matrix and increased production of pericellular hyaluronic acid) to facilitate the invasion of neoplastic cells. Also, fibroblasts/myofibroblasts may aid in the induction of epithelial mesenchymal transitions (EMT) of malignant cells (15). Thus, TD-exosomes typically

increase the growth and progression of primary neoplastic lesions via the induction of phenotypic changes that facilitate more aggressive cellular characteristics.

Exosomes can provide an efficient and specific transfer of molecular signals between cells. If these signals induce oncogenic changes in the cellular phenotype, TD-microvesicles are sometimes referred to as “oncosomes”. A variant of the epidermal growth factor receptor, EGFRvIII, has oncogenic features via unregulated stimulation of the Akt/MAPK pathway which, for example, increases VEGF. Microvesicles from xenografts of glioblastomas have been reported to transfer EGFRvIII to glioma cells lacking this phenotypic feature. Because the EGFRvIII expression remained stable in malignant cells to which EGFRvIII had been transferred, such transfer of oncogenic features could be a mechanism via which oncogenic features can horizontally spread among cells. In response to continuing exposure to exosomes, endothelial cells also transiently developed an EGFRvIII expressing phenotype resulting in increased growth and hence, probably increased angiogenesis. The phenotypic features of endothelial cells associated with increased levels of EGFRvIII were not stable and ended when the exposure to the TD-exosomes containing EGFRvIII ceased (~~79-81~~90-93).

Induction of an oncogenic phenotype via larger TD-microvesicles isolated from the MDA-MB-231 breast cancer cell line and the U87 glioma cell line also has been reported. Microvesicles released from these cell lines contained both tissue transglutaminase (tTG) and fibronectin, a tTG substrate. These microvesicles caused NIH/3T3 fibroblast and benign breast epithelial cells, MCF10A, to develop some aspects of malignant behavior such as anchorage - independent growth in agar and growth in medium with low concentrations of serum. Maintenance of these

features of malignant behavior in non-malignant cells also required continuous exposure to TD-microvesicles (114,115~~00,101~~). Local effects of exosomes on primary neoplastic lesions are demonstrated in Figure 2.

Exosomes facilitate metastases: How and where tumors metastasize are affected by multiple molecular features including functioning genes in primary tumors which stimulate or inhibit metastases (116~~02~~). TD-exosomes from primary tumors promote the metastatic spread of tumors by inducing features at potential metastatic sites which attract neoplastic cells and build a matrix for their attachment, aid neoplastic cells at the metastatic site to survive, stimulate angiogenesis and suppress immunity (19-22). Thus, there is likely a role of exosomes in the release of their chemotactic contents or the stimulation of non-neoplastic cells to release chemotactic molecules in attraction of malignant cells to potential sites of metastasis.

TD-exosomes not only induce molecular features that cause malignant cells to leave the primary tumor, and via chemotaxis facilitate the migration of neoplastic cells to sentinel lymph nodes where exosomes establish a supportive environment that promotes metastases. Specifically, exosomes from melanomas stimulate the production of $\alpha_v\beta_3$ integrin, ephrin receptor β4, and stabilin 1 which act to recruit melanoma cells to the ipsilateral sentinel lymph node. Exosomes from melanomas also induce the production of collagen 18, laminin 5, MAP kinase (p38) and urokinase plasminogen activator protease which aid in the production of the matrix necessary to support the growth of metastatic cells. In addition, exosomes also increase nodal metastases by inducing angiogenesis via inducing the expression of vascular endothelial growth factor β (VEGFβ), TNFα, and hypoxia inducible factor 1α (19); similarly, exosomes affect

the programic development of some bone marrow derived cells (BMDC) via upregulation of MET to produce a "pro-vasculogenic phenotype" which facilitates angiogenesis and metastasis (21,22). Figure 3 demonstrates some of the features that facilitate metastases to ipsilateral lymph nodes.

TD-exosomes may interact with soluble factors from tumors to facilitate metastases. For example, when the conditioned media from cultures of cells of a syngenic pancreatic tumor model was separated into exosomal and soluble components, exosomes were more effective than the soluble factors in increasing metastases to lymph nodes and/or lung; however, the combined soluble and exosomal fractions were more effective in increasing metastases (20). The facilitation of metastases by exosomes was greatly decreased when there was knockdown of CD44 splice variants, especially CD44v6 on which production of matrix depends. This matrix which supports the attachment and growth of metastatic cells contained c-Met and urokinase-type plasminogen activator receptor (20) which have been reported to increase metastases (21,22).

Much of the work on how exosomes affect metastases is based on the responses of melanomas to TD-exosomes/microvesicles from cell lines grown *in vitro* in standard 2D cultures. There needs to be increased characterization of the effects of exosomes *in vivo* in providing autocrine, paracrine and endocrine signals that modulate the growth, progression and metastases of tumors other than melanomas.

TD-E~~xosomes increase the resistance of tumors to therapies~~~~XOSOMES INCREASE THE RESISTANCE OF TUMORS TO THERAPIES~~: Exosomes ~~aid~~ normally function in the export of

waste products and less needed ~~unneeded~~ molecules from cells as well as many harmful products such as exogenous molecules~~chemotherapeutic and other drugs~~ (4,23,49). In cancers, exosomes also function to export chemotherapeutic drugs such as c~~C~~is-platinum and doxorubicin; ~~are exported by TD-exosomes and~~ thus, TD-exosomes are utilized as a pathway of chemoresistance of specific malignant cells to specific drugs. All drugs are not exported from cells. For example, ~~In contrast,~~ TD-exosomes do not export 5-fluorouracil efficiently. When factors associated with the shedding of exosomes were combined into a "vesicle shedding index", the vesicle shedding index of the "NCI 60-cell-line panel" was found to be positively correlated with 50% growth inhibition (GI_{50}) for most of the 171 compounds of the NCI "Standard Anticancer Agent Database". Also, the actual shedding rate of microvesicles from 6 cell lines correlated positively with doxorubicin resistance; however, there was no correlation of vesicle shedding with chemoresistance to 5-fluorouracil (23).

The secretion of drugs in exosomes such as doxorubicin may involve vacuolar protein sorting 4a (VPS4a) which is important in secretion of exosomes. Specifically, disruption of VP54a in the erythroleukemic cell line, K562, inhibited the efflux of doxorubicin (115~~103~~). Thus, exosomes are likely to be a major factor in the chemoresistance of malignant cells to a variety, but not all, chemotherapeutic drugs.

THE POTENTIAL CLINICAL IMPACT OF EXOSOMES*:*

The molecular features of TD-exosomes mirror many of the molecular features of the tumors from which they arise. TD-exosomes have been reported to contain biomarkers characteristic of tumors including those of the bladder, brain, colorectum, kidney and melanomas so the

presence of biomarkers characteristic of tumors in exosomes of biological fluids may aid in clinical decisions and in translational research on biomarkers; thus~~. Thus~~, TD-exosomes may be important ~~in translational research and/or~~ in clinical decisions including risk assessment, early detection, and diagnosis as well as in the prediction of therapeutic efficacy, in determining prognosis, and as surrogate endpoints in evaluating chemotherapeutic, preventive, and novel therapies. Of special importance in translational research is that exosomes are carried in blood and are shed into biological fluids such as ascites and pleural fluids, all of which can be obtained easily for clinical use. As discussed, the measurements of biomarkers in exosomes from bodily fluids are likely to be just as sensitive and specific as measurements of the same biomarkers in the matching bodily fluids. When specific biomarkers or biomarker panels (signatures) are more concentrated in exosomes, their measurements in the exosomal fraction may be more effective in solving clinical problems than their measurements in matching bodily fluids (90-93~~78-81~~). However, because exosomes and microvesicles and their contents are essentially invisible, use of the exosomes/microvesicles in translational research is in its infancy.

A biomarker of ovarian epithelial tumors, Claudin 4, has been found to be increased in exosomes separated from the blood of patients with ovarian carcinoma (118~~104~~) and TMPRSS2-ERG, δ-catenin, and PCA-3 which are potential biomarkers for cancers of the prostate, have been measured in TD-exosomes isolated from the urines of prostate cancer patients (119~~05~~). Similarly, microRNAs may aid in the early diagnosis of cancers of the lung via their measurement in TD-exosomes (120~~06~~). Biomarkers measured in exosomes also may be useful in measuring responses to therapy.

Some TD-exosomes that contain increased concentrations of specific tumor antigens could be used to stimulate DCs to secrete exosomes that initiate cytotoxic T-lymphocytic responses (5,6). The generation of exosomes by DCs *in vitro* would be an approach that could avoid the negative effects (e.g., reduction of immunity) of using TD-exosomes *in vivo*. To date, the clinical results of using exosomes in cancer therapy have been modest, but there have been few major side effects (10-12).

Because of the suppression of immune surveillance by TD-exosomes, TD-exosomes could be targeted to reduce the suppression of immunity in patients with cancers. Strategies have been proposed to increase immune reactions to cancer by reducing the transfer to TD-exosomes of molecules that suppress the immune system. Alternatively, molecules which may stimulate immune cells might be introduced into TD-exosomes. Similarly, the function of TD-exosomes in cellular export of drugs could be targeted to increase the effectiveness of many drugs by reducing the release of TD-exosomes via changing local pH or targeting the pathways involving TD-exosomes such as the VSP4a signaling pathway (10-12,~~103,107~~117,121). TD-exosomes also could be decreased in blood using a "hemopurifier" that selectively removes TD-exosomes from blood by immobilized antibodies that bind to the surface molecules of TD-exosomes.

Because TD-exosomes can be taken up specifically by neoplastic cells, they could deliver drugs, preventive agents, small molecules (e.g., microRNAs), and agents of gene therapy to the cells of specific tumors (122-126). Curcumin is a natural product with anti-tumor and anti-inflammatory features. Whose bioavailability and therapeutic efficacy is limited due to poor solubility. Its clinical usefulness is improved by the incorporation of curcumin into exosomes which were

much more effective than liposomes containing curcumin in preventing septic shock in a murine model (~~108-110~~122-125). ~~This demonstrates how~~Both drugs and microRNAs could be ~~targeted~~ delivered specifically by exosomes to neoplastic cells (~~111~~125,126)

The clinical uses of microvesicles are being studied by commercial as well as academic organizations as vehicles to deliver chemotherapy, small molecules, agents of gene therapy and/or prevention to target cells more specifically than systemic administration. Thus, multiple approaches to the selective delivery of therapeutic and preventive agents via biological microvesicles are in active development.

Summary:

Exosomes and related microvesicles comprise a newly identified method of local and distant intercellular communication. Tumors have hijacked this mechanism of intercellular communication to aid in their growth, progression and dissemination. TD-exosomes can act to produce a fertile environment to support growth at primary sites of neoplastic lesions and potential sites of metastases. TD-exosomes also may facilitate tumor growth and dissemination by inhibiting immune surveillance as well as by increasing chemoresistance via removal of chemotherapeutic drugs. Thus, TD-exosomes might be potential targets for therapeutic interventions via their modification or removal. Exosomes and related microvesicles also could serve as specific delivery vehicles to neoplastic lesions of drugs, small molecules or agents of prevention and gene therapy. In other clinical approaches, TD-exosomes could serve as a sub-compartment in which biomarkers could be measured to aid in the early detection and diagnosis of diseases, in determining prognosis, in prediction of therapeutic efficacy and in

determining therapeutic responses. Such approaches would utilize the molecular features of TD-exosomes that are different from exosomes of either associated or diseased controls, or from normal individuals.

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Table 1 – Source of exosomes in a patient with a specific malignant process

Independent of the tumor

- Exosomes from normal cells
- Exosomes from cells whose release is caused by non-neoplastic comorbid conditions that are independent of the tumor

Dependent on the presence of cancer

- TD-exosomes from malignant cells
- Exosomes from uninvolved cells (e.g., adjacent uninvolved tissue that appears normal) induced by TD-exosomes
- Exosomes from normal cells induced by exosomes or other molecules form the cancer (e.g., soluble factors). This includes exosomes released from tumor, associated stromal cells.
- Exosomes from immune cells affected by the cancer
- Exosomes from the cells of a co-morbid condition caused by the tumor (e.g., disseminated intravascular coagulation caused by the cancer)

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Legend – Figure 1

The scheme in Figure 1 summarized some of ~~Figure 1 demonstrates~~ the complex effects of TD-exosomes on the immune system and how TD-exosomes suppress immune surveillance by decreasing the proliferation and activation of T and NK cells, decreasing mature antigen presenting cells and increasing cells such as myeloid derived suppressor cells and Tregs which suppress immunity. HSP72 – heat shock protein 72; IL-6, IL-10, interleukins 6 and 10; JAK3 – Janus kinase 3; MyD88-myeloid differentiation primary response 88; PGE2 – prostaglandin E2; pStat3 – phosphorylated signal transducer and activator of transcription 3; TGFβ – transforming growth factor β; TNF-α – tumor necrosis factor alpha.

Legend – Figure 2

A scheme indicating the potential~~Figure 2 demonstrates the~~ local effecs of TD-exosomes on cancer cells is presented in Figure 2. The divisions of boxes represent, for example, potential different exosomal compartments that interact with the microenvironment of the tumor. Paracrine networks are represented between fibroblasts, myofibroblasts and malignant cells.

Legend – Figure 3

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AFigure 3 presents a model for the effecs of TD-exosomes on metastasis. The model is based on studies of metastases of melanomas is shown in Figure 3. Abbreviations are as follows: HSP72 – heat shock protein 72; IL-6, IL-10, interleukins 6 and 10; JAK3 – Janus kinase 3; MyD88-myeloid differentiation primary response 88; PGE2 – prostaglandin E2; pStat3 – phosphorylated signal transducer and activator of transcription 3; TGFβ – transforming growth factor β; TNF-α – tumor necrosis factor alpha.

Genome-Wide MicroRNA Profiling of Novel African American and Caucasian Prostate Cancer Cell Lines Reveals that *miR-152* is Silenced by DNA Methylation

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Keywords:
miRNA, DNA methylation, African American, prostate cancer

Abbreviations:
AA, African American
CA, Caucasian
miRNA, microRNA

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Abstract

Background: African American (AA) men have almost twice the incidence and death rates related to prostate cancer compared to Caucasian (CA) men. Some of these differences may be attributed to the elevated expression of different genes; however, the expression of microRNAs (miRNAs) has not been widely explored. Therefore the aim of this work is to determine the miRNA expression profile of novel AA and CA prostate cancer cell lines and correlate with clinical progression of disease.

Results: With a novel panel of AA- and CA-derived cell lines, hierarchical clustering and partial least squares plots demonstrated a distinct miRNA signature in AA cell lines, independent of tumor status. Evaluation of the most differentially expressed miRNAs showed that miR-132, miR-367b, miR-410, and miR-152 were decreased in more aggressive cells and that this expression was reversed after treatment of the cells with the demethylating agent, 5-aza-2′-deoxycytidine. Bisulfite conversion and sequencing of the miR-152 promoter showed that it was highly methylated in LNCaP, and PC-3 cells. Ectopic expression of miR-152 resulted in decreased growth, migration, and invasion. Informatics analysis of a large patient cohort showed that decreased miR-152 expression correlated with increased metastasis and biochemical recurrence. In a panel of 39 prostate cancer tissues with matched controls (20 AA and 19 CA), tumors from 50% of AA patients and 35% of CA patients had statistically significant lower miR-152 expression. Ectopic expression of miR-152 in both LNCaP and PC-3 cells down-regulated DNA (cytosine-5)-methyltransferase 1 (DNMT1) through direct binding in the DNMT1 3'UTR. There appeared to be an inverse relationship, as cells treated with DNMT1 siRNA re-expressed miR-152.

Conclusion: In summary, these results are the first to identify unique miRNAs that contribute to aggressive prostate cancers in AA patients. Furthermore epigenetic regulation of the miR-152/DNMT1 regulatory loop may play an import ant role in multiple events that contribute to the aggressiveness of PCa tumors.

Introduction

The most commonly diagnosed type of cancer among men in the US is prostate cancer (PCa), which accounts for 29% (241,740) of all new cancer cases. Although the number of new cases of PCa has decreased in recent years, there are still racial and ethnic differences in PCa epidemiology. African-Americans (AAs) have the world's highest incidence of PCa and more than twofold higher mortality rate compared with Caucasian Americans (CAs) [1]. Overall, AA patients are younger and have higher Gleason scores, PSA levels, and incidence of palpable disease [2]. Various factors have been associated with the more aggressive prostate tumors. For example, differential gene expression in AA patients contributes to aggressive disease [3-7], and epigenetic mechanisms, such as DNA methylation, result in the loss of key regulatory genes [8, 9]. This is particularly evident for AA patients where hypermethylation of genes in normal or pre-malignant areas are thought to predispose to malignancy [10, 11]. However, the underlying mechanism of these acquired methylation patterns is poorly understood.

MicroRNAs (miRNAs) are small RNA molecules consisting of 19–23 nucleotides that regulate various biological processes. More than 60% of protein-coding genes may be targeted by miRNAs [12], mainly through translational repression and degradation of target mRNAs. An expanding body of evidence supports a role for miRNAs in disease progression and the potential for epigenetic mechanisms, such as DNA methylation, to regulate miRNA expression [13, 14]. Recently, DNA methylation of proximal CpG islands in miRNA promoters was described as a method for decreased expression in various cancers, including PCas [15-18]. Although miRNAs are expressed differently in healthy tissues and cancers [19] and in localized and advanced tumors [20], little is known about racial differences in miRNA expression. Identification of unique miRNAs and mRNA-associated targets will begin to clarify the specific events involved in the progression of PCas in AAs.

To address this question, our laboratory has established non-malignant and malignant cell lines derived from AA PCas that replicate many of the clinical features of the original PCas [21, 22]. Thus, utilizing these cell lines and commonly available PCa cell lines, we explored the possibility of race-related differences in miRNA expression. Herein, we report that AA cell lines have a distinct miRNA signature, independent of tumor status. Evaluation of the miRNAs most differentially expressed demonstrated that miR-132, miR-367b, miR-410, and miR-152 were decreased in more aggressive cells and that this expression was reversed after treatment of the cells with 5-aza-2′-deoxycytidine (5-aza-2'd). Bisulfite conversion and sequencing of the promoter showed that miR-152 was highly methylated in LNCaP, DU-145, and PC-3 cells. Ectopic expression of miR-152 resulted in decreased cell proliferation, migration, and invasion. In a panel of 39 PCa tumors with adjacent matched controls (20 AA and 19 CA) , 50 % of AA patients and 35% of CA patients demonstrated statistically significant lower miR-152 expression compared to adjacent controls. Ectopic expression of miR-152 down-regulated DNA(cytosine-5)-methyltransferase 1 (DNMT1) through direct binding in the DNMT1 3'UTR. This appeared to be an inverse relationship, as cells treated with DNMT1 siRNA re-expressed miR152. Finally, as determined with a large patient cohort, loss of miR-152 was related to poor clinical outcomes.

Results

miRNA Profile of Panel of AA and CA Cell Lines

To determine the miRNA expression pattern in our panel of AA and CA prostate cell lines, the expression of 662 miRNAs was analyzed utilizing Asuragen Affymetrix Gene Chips. To validate our model, cell lines were first grouped by malignancy. Hierarchical clustering and a PLS plot of cell lines demonstrated distinct expression patterns in the malignant and non-malignant cell lines (Figure 1a), with 21 differentially expressed miRNAs between the groups (Supplemental Table 1 ($p<0.00001$). Utilizing racial background as criteria, distinct hierarchical clustering of miRNAs was evident between the AA and CA cell lines (Figure 1c), regardless of malignancy status. A PLS plot reflected this pattern, with distinct variations in the racial profiles of the AA and CA cell lines (Figure 1d). In this variation, there were 47

miRNAs differentially expressed by race (Supplemental Table 2). The 11 most significant race related miRNAs ($p<0.00001$) are listed in Table 1. To confirm these race related miRNAs, we interrogated a microarray dataset that consisted of primary tumor cells isolated from 5 AA and 4 CA patients (Supplemental figure 4). The significant race related miRNA probes were able to differentiate patients relevant to race using PLS test (Supplemental Figure 1).

Lastly, of the 47 miRNAs significantly expressed, we confirmed the 5 most significant by qRT-PCR in non-malignant and selected aggressive malignant cell lines (Supplementary Figure 2). Of these, miR-132, miR-376b, miR-410 and miR-152 had an expression profile correlating with functional features of the metastatic cell lines.

Demethylation Treatment Reverses miRNA Expression of AA-Associated miRNAs.

Previous reports and *in silico* analyses demonstrated that most of the miRNAs associated with race contain CpG islands within the promoter regions upstream of the start site (Supplemental Figure 3). To determine if hypermethylation is associated with decreased expression of these miRNAs, LNCaP and PC-3 cells were treated with 5 μM 5-aza-2'd alone for three days or in combination with 100 nM TSA for 24 hr. Re-expression of multiple miRNAs was evident in both LNCaP and PC-3 cells after 5-aza-2'd treatment. However, miR-376b and miR-152 showed the most substantial increases in both cell lines (Figure 2).

Since miR-152 contained the greatest percentage of methylated CG sequences and demonstrated the most consistent increases after 5-aza-2'd treatment, we focused on this RNA. To determine the methylation status of the miR-152 promoter region, extracted DNA from LNCaP, DU-145, and PC-3 cell lines was subjected to sodium bisulfite modification and sequenced. To confirm these results, sodium bisulfite-converted DNA was subjected to capillary electrophoresis and analyzed utilizing the online Bisulfite Sequencing DNA Methylation Analysis (BISMA) sequencing program. The results indicated that DNA of LNCaP, DU-145, and PC-3 cell lines was 100% methylated at 1000 base pairs upstream

from the promoter region (Figure 3). Thus, these results suggest that, in malignant PCas, miR-152 is inactivated through methylation.

miR-152 Expression Correlates with Clinical and Pathological Variables.

The expression of miR-152 in 28 normal cell lines, 97 primary tumors, and 13 metastases was analyzed using the Taylor et al. GSE21032 data set available on the GEO website (http://www.ncbi.nlm.nih.gov/geo/). Primary and metastatic tumors had lower levels of miR-152 relative to normal samples (Figure 4a), which correlated with the higher incidence of metastatic samples, metastatic events, and lymph node invasion (Figures 4b and c). Tumors with low miR-152 levels also had reduced biochemical recurrence-free survival (Figure 4c). Together, this describes a consistent picture of low miR-152 levels associated with PCa metastasis and recurrence.

These results prompted us to determine miR-152 expression in our patient cohort of AAs and CAs, hypothesizing that tumors from AAs would have lower miR-152 than those from CA patients. Patients were selected based on cancers of higher total Gleason score (≥6) and/or pathological stage (pT2), and positive for perineural and/or vascular invasion, as these pathological characteristics correlate positively with tumor aggressiveness and metastasis. AA patients had a lower median age relative to similarly staged CA patients, which was also associated with lower miR-152 levels measured by qRT-PCR (Table 2). Analysis of miR-152 expression in individual tumors compared to matching adjacent normal controls showed a statistically significant decrease in miR-152 expression in 50% of the AA patients compared to only 35 % of CA patients (Figure 5a, b supplemental table 3).

Restoring miR-152 Expression Decreases Cell Growth.

The clinical relevance of miR-152 in PCa prompted us to determine if loss of expression had a biological or functional role in promoting metastatic tumors. After optimizing the concentration of miR-152 mimics that restored miR-152, miR-152 was transfected into LNCaP and PC-3 cells. As determined by MTT assays, ectopic expression of miR-152 inhibited cell proliferation after 3 days, and this continued to Day 6 (Figure 6a). Since the inhibition of cell proliferation began at 72 hr, miR-152 transfected cells

were next assayed after 72 hr by flow cytometry to determine if there was an effect on cell cycle progression. miR-152 treatment caused cells to accumulate at the G2-M phase (LNCaP NC 34.50% compared to miR-152-transfected cells 35.25%; PC-3 NC=33.15% vs miR-152= 37.59%) (Figure 6 b,d). In addition to reduced cell proliferation, miR-152 transfected LNCaP and PC-3 cells demonstrated a decrease in cell migration (Figure 6e) and invasion (Figure 6f).

Previously, miR-152 was demonstrated to target DNMT1 expression in endometrial tumors [37]. Since we observed that numerous miRNAs in our panel were influenced by methylation, and the methyltransferase DNMT1 is a regulator of DNA hypermethylation in various tumor types, we sought to explore the miR-152/DNMT1 relationship in PCas. First, the relative expressions of DNMT1 and miR-152 were examined in our panel of cell lines. As expected, qRT-PCR showed that DNMT1 expression was elevated in the more aggressive cell lines, and this correlated with decreased expression of miR-152 (Figure 7a). DNMT1 expression was then examined in LNCaP and PC-3 cells transfected with miR-152. Both types of cells showed decreases in DNMT1 expression at the RNA and protein levels (Figure 7b). This relationship is possibly direct, as miR-152 has putative binding sites in position 45-54 of the DNMT1 3' UTR. To confirm this, we utilized a previously established DNMT1 3′-UTR luciferase reporter system [38] containing the miR-152-binding sites (DNMT1 wild-type 3′- UTR) or mutating these sites (DNMT1 Mu 3′-UTR) that contain the putative miR-152 binding sites. Both LNCaP and PC-3 miR-152 transfected cells showed decreased DNMT1 wild-type 3′-UTR luciferase activity, whereas DNMT1 Mu 3′-UTR luciferase activity was not affected (Figure 7c). Since we demonstrated that loss of miR-152 occurs through promoter methylation, we asked if there was a reciprocal relationship between miR-152 and DNMT1. Treatment of LNCaP and PC-3 cells with DNMT1 siRNA caused increases in miR-152 expression (Figure 7d).

To examine the possible broader influence that loss of miR-152 has in PCas, we queried the TargetScan *in silico* database to determine additional gene targets that could be regulated by miR-152. Of the top genes, Rictor, TGF-β, SOS1, ABCD3, SMAD4, SOX2, E2F1, and Dicer were predicted. To

determine their influence, a custom gene array of these genes was designed (Supplemental Table 5), and expression levels in LNCaP and PC-3 cells that were transfected with miR-152 were assayed. Validation of the down-regulated targets by qRT-PCR and immunoblots indicated that, in addition to DNMT1, Rictor and SOS1 were influenced by miR-152 in LNCaP and PC-3 cells; however, to a lesser extent in PC-3 cells (Supplemental Figure 4 a-c). Together, these results indicate that miR-152 regulates epigenetic events that promote tumorigenesis.

Discussion

Numerous studies have now reported gene differences AA and CA prostate tumors [4, 6, 7, 18, 39]. As the field of health disparities is in its infancy, these reports provide the first evidence of population-based genetic contributions to aggressive disease. However, the regulation of these genes is still elusive. To address this question, without influence of non-epithelial stromal cells typically present in tissues, we utilized AA- and CA-derived cell lines derived from benign and primary tumors, along with well-established cell lines derived from metastatic PCas. Our findings derived by miRNA microarray analysis revealed a distinct miRNA expression pattern in AA-derived cell lines. qRT-PCR validation of these miRNAs showed that the five most significant miRNAs were decreased in metastatic cell lines, suggesting a role for these molecules in tumorigenesis. Since previous reports have demonstrated inheritable epigenetic changes in AA patients, we sought to determine if decreased expression was the result of DNA methylation. *In silico* analysis revealed that these miRNAs have dense CG regions in the promoter. Further evidence that methylation of CpG islands causes silencing is that each of the miRNAs, albeit to varying degrees, is re-expressed after treatment with 5-aza-2'd or TSA. To our knowledge, this is the first report that demonstrates a methylated miRNA profile that can be associated with the progression of AA prostate tumors.

Of the miRNAs influenced by 5-aza-2'd, several have been found in other tumor types, including PCa. For example, miR-132, which targets both heparin-binding epidermal growth factor and TALIN2, is silenced by hypermethylation [15]. Of the miRNAs we assayed, miR-152 demonstrated the most

significant reversal of expression, an effect consistent for both LNCaP and PC-3 cell lines. Previous reports regarding endometrial cancer [37], gastrointestinal cancer [40], and ovarian cancer [41, 42], and a report relating to PCa [43], published during the preparation of this manuscript, show that miR-152 levels are decreased in more advanced tumors. Analysis of patient data from the Taylor et al. study, deposited in the NIH-sponsored Geo database, confirms that miR-152 expression is low in the more advanced primary tumors, with the lowest expression in metastatic samples. However our analysis provides evidence that low miR-152 levels decreases probability of biochemical recurrence free survival in patients. qRT-PCR to measure normal-tumor expression ratios confirmed these findings, with 67% of patients displaying lower miR-152 expression. However, the most relevant observation is that AA patients display decreased expression of miR-152 in both uninvolved and paired tumors compared to CA patients of similar age, stage, and Gleason grade. To determine the mechanism for decreased miR-152, sodium bisulfite modification and sequencing of miR-152 promoter in aggressive PCa cell lines were performed. Both LNCaP and PC-3 cells showed 100% methylation status, highlighting that, in aggressive tumors, hypermethylation of the miR-152 promoter is a mode of silencing miR-152.

miR-152 directly targets the 3′ UTR of DNMT1 [37]. Across our cell line panel, DNMT1 and miR-152 showed an inverse relationship in expression. With LNCaP and PC-3 cells, which have endogenously low miR-152 levels, we confirmed that forced expression of miR-152 results in decreased expression of DNMT1 at both the RNA and protein levels. We also confirmed decreased expression of several candidate targets that have been implicated in AA prostate tumors. SOS-1, which is a regulator of EGFR expression and downstream signaling, is increased in AA PCas [5]. Although Rictor, a subunit of the mTORC2 complex, has not been directly implicated in AA PCas, the phosphatidylinositol-3-kinase/AKT (PI3K/AKT) pathway, which is a regulator of the mTORC2 complex, has been associated with AA PCas [39]. Whereas TGF-α is a direct target of miR-152 in PCa cell lines [43], we observed decreased TGF-β mRNA expression after forced miR-152 expression, which further suggests that loss of miR-152 promotes increased aggressiveness through multiple signaling pathways. Because DNMT1 has been characterized as one of the main enzymes responsible for maintenance of methylation patterns in

tumor-related genes [44, 45], we investigated the role of DNMT1 on miR-152. We found that depletion of DNMT1 through siRNA resulted in increased miR-152 in both LNCaP and PC-3 lines, highlighting that the effect is independent of androgen sensitivity. However, there appears to be a feed-forward loop where either loss of miR-152 and/or increased DNMT1 maintains methylation patterns, and hence miR-152 expression. MiR-152 is gaining interest as a factor in various tumor types; a recent report concerning nickel sulfide-transformed human bronchial epithelial (16HBE) cells demonstrated that the miRNA-152/DNMT1 relationship develops early in transformed cells [38]. Treatment of cells with 5-aza-2'-d or depletion of DNMT1 led to increased miR-152 expression by reversal of promoter hypermethylation. These findings are similar to those presented here, however the available data relating to PCas suggest that the relationship between loss of miR-152 and increased DNMT1 occurs during the progression to advanced tumor status.

Our finding of a regulatory mechanism that maintains methylation patterns has implications for AA PCa patients. The fact that miR-152 expression is lower in non-malignant tissues from AA patients compared to CA non-malignant tissues suggests that miR-152 expression is regulated by inheritable differences. Although the cause of this decreased expression is still speculative, using NCBI Genome and dpSNP Database (http://www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi?rs=12940701), we did identify 38 single-nucleotide polymorphisms (SNPs) on chromosome 17q21 (46114527…..46114613, complement1) region where miR-152 is located. Of these, rs200114569, which is associated with a C to G nucleotide change, and rs12940701, which is associated with a C to T change, were of interest because they may affect the methylation status of the miR-152 gene. The rs12940701 SNP has a relatively high frequency (15%) of the minor T allele in the European American population (n=120) relative to the Yoruba (Nigerian) population (n=118). Thus, the European American population could have lower methylation rates since the C nucleotide is changed to the T nucleotide, whereas, in the Yoruba population, this shift is less frequent, allowing for more C nucleotides, thus possibly increasing the rate of methylation. Further analysis should focus on the methylation frequency of miR-152 and on genotyping SNPs that compromise miR-152 expression in AAs and CAs.

In summary, the present work highlights miR-152 as a tumor suppressor that is inactivated by methylation. Because a large number of tumor/metastasis suppressor genes are silenced as a result of methylation, miR-152 could be a central regulator of key events that contribute to tumorigenesis and aggressiveness and thus has the potential to be a therapeutic agent for PCa treatment. These results begin to unravel the molecular mechanism associated with the aggressive tumors of AA patients.

Materials and Methods

PCa cell lines and primary tissue samples. Immortalized PCa cell lines RC-77T/E (T3c poorly differentiated primary tumor), RC-77N/E (non-malignant) were derived from AA patient as previously described [21]. RC-43T/E (T4 poorly differentiated primary tumor) and RC-43N/E (non-malignant) were also obtained from an AA patient (characterization unpublished). Benign cell lines derived RC-165N/hTERT (derived from AA patient) [23] and RC-170N/hTERT (derived from CA patient) [24], and primary tumor cell line RC-92a/hTERT (derived from CA patient) cells [25]. All cell lines were cultured in keratinocyte serum-free medium (KGM, LifeTechnologies, Carlsbad, CA) supplemented with bovine pituitary extract, recombinant epidermal growth factor, and 1% penicillin-streptomycin-neomycin were maintained in KGM medium as previously described [26]. Non-malignant CA prostate epithelial cells (PrEC) were obtained from Clonetics Lonza (Switzerland) and maintained in Prostate Epithelial Cell Growth Medium (Clonetics). The CA androgen-independent and metastatic PC-3 and DU-145 PCa cell lines were maintained in Dulbecco's Modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin-neomycin. The CA LNCaP and C4-2b androgen-dependent and -independent, respectively, prostate cells were maintained in T-medium. RWPE-1 normal prostate cells were maintained in KGM as previously described [27]. The malignant, androgen-receptor positive AA MDA-PCa-2b cells were purchased from ATCC (Manassas, VA). These cells were maintained in F-12K medium supplemented with 20% fetal bovine serum, 25 ng/ml cholera toxin, 10

ng/ml mouse epidermal growth factor, 5 μM phosphoethanolamine, 100 pg/ml hydrocortisone, 45 nM selenious acid, and 5 μg/ml bovine insulin.

Patient Samples. Patient samples were obtained from the Cooperative Human Tissue Network at the University of Alabama at Birmingham (UAB) under Institutional Review Board-approved protocols. Additionally, the Institutional Review Boards of Tuskegee University and UAB approved the use of tissues for this study. Tumor sections were macro dissected. There was no significant difference between the two groups with respect to tumor content. (Average percentages of tumor for AA and CA biopsies were both 54%.) Paired normal tissues were also collected from the same patients. Altogether, samples from each of the 20 AA and 19 CA patients, along with patient-matched normal adjacent tissues, were used for qRT-PCR validation. p-values were generated using the Benjamini-Hochberg corrected for multiple tests that reflects the individual patients with significant difference in mir152 expression relative to global mean of u48 for Ct values and global mean for delta Ct values.

Quantitative Real-time PCR (qRT-PCR). Total RNA extraction was performed using the Ambion recover all nucleic acid isolation kit (AM 1975) modified by replacing filters with (AM10066G). RNA (10 ng) was reverse transcribed using TaqMan miRNA reverse transcription kits (Life Technologies). For mRNA expression, 1 μg of total RNA was reverse transcribed using High Capacity cDNA kits (Invitrogen). Relative expression of miRNAs and mRNA was quantified with the TaqMan Universal PCR Master Mix, No AmpErase UNG, with the 7500 Fast Real-Time PCR system (Life Technologies). Thermal cycling conditions included enzyme activation for 10 min at 95°C, 40 cycles of 95°C for 15 s, and 60°C for 60 s, according to provider's protocol. Reverse transcription for mRNA was accomplished as previously described [22, 28]. SYBR Green reagents (Invitrogen) were used for quantitative real-time PCR. Thermal cycling conditions for primer sequences used for mRNA detection are included in the table below . Analyses for miRNA and mRNA were performed in triplicate. RNU48 miRNA, GAPDH, and 18S ribosomal RNA were used as endogenous controls.

Transfection of miRNA Precursor. LNCaP and PC-3 PCa cell lines were seeded at 2 x 10^5 cells in six-well plates on the day before transfection. Lipofectamine 2000 reagent (Life Technologies) was used to transfect 5-15 nmol of miR-152 or 5-15 nmol of miR-Negative Control #1 in Opti-MEM (Life Technologies). Cells were harvested for assays three days after recovery in fully supplemented media, as described previously [28].

Cell Proliferation Assays. LNCaP and PC-3 cells (2.5 x 10^2) were plated in 96-well plates in DMEM. After being cultured for 24 hr, cells were transfected with miR-152 or Negative Control #1 (Life Technologies) at a concentration of 30 nM. Cell viability was determined at 24, 48, 72, 96, and 120 days after transfection. All cells were incubated with 10 µl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich, St. Louis, MO) solution (5 mg/ml) in PBS for 4 hr. Dimethylsulfoxide (50 µl) was added for 10 min after aspiration of the MTT solution. Plates were read at 560 nm.

Cell Migration and Invasion assay. Cell migration and invasion was determined using the Boyden chamber assay. Briefly, 20,000 cells were plated in the upper chamber, with or without Matrigel, containing serum-free medium containing 1% bovine serum albumin for 24 hours; this then was replaced with a serum-free medium for an additional 24 hours. The lower chamber contained medium with 10 ng/mL of epidermal growth factor was used as a chemo-attractant. The number of cells that migrated or invaded through the matrix over a 48 hour-period was determined by counting cells that stained with crystal violet on the bottom of the filter. All experiments were performed in triplicate.

Sodium Bisulfite Modification and Methylation Analysis. DNA was extracted with GenElute Mammalian Genomic DNA Miniprep kits (Sigma-Aldrich) following the manufacturer's instructions. Subsequently, bisulfite conversion of DNA (2 µg) was performed with EpiTect Bisulfite kits (Qiagen, Valencia, CA). The miR-152 sequence was determined by using the UCSC Genome Browser [30].

Methylation PCR amplification primers for sequencing were designed using MethPrimer [31]. Sequence *from 5' to 3'* - Primer 1: GGGTTAGGGGGAGTAGTTAATTTAG and Primer 2: ATAAACTCCAAAAACATACCCATCA. qRT-PCR was performed on bisulfite-converted DNA (1 μl), using the primers described above covering two CpG regions upstream from the miR-152 promoter DNA sequence. A second round of amplification was performed on the first PCR amplicons and subsequently characterized by electrophoresis on 1.2% agarose gels. Sequencing of amplicons was performed at the UAB Heflin Center, Birmingham, AL. Bisulfite sequencing was analyzed by an online DNA methylation platform, Bisulfite Sequencing Data and Presentation Compilation (http://biochem.jacobs-university.de/BDPC/).

Immunoblots. Cells were harvested 3 days after miR-152 transfection, washed with PBS, and lysed in NP-40 lysis buffer [10 mM Tris-Cl (pH 7.4), 10 mM NaCl, 3 mM $MgCl_2$, 0.5% NP-40 (Nonidet P-40), 0.15 mM spermine, 0.5 mM spermidine, and protease inhibitor solution] and centrifuged at 12,000 g for 10 min at 4°C. Protein concentrations were determined with the BCA Protein Assay kit (Thermo Scientific, Rockford, IL) according to manufacturer's instructions. Protein samples were separated on 7.5% SDS-PAGE pre-cast gels (Thermo Scientific). Protein levels were detected by anti-DNMT1, anti-mTOR, and anti-Rictor rabbit polyclonal antibodies (Cell Signaling, Danvers, MA), and analyzed by chemiluminescence.

Treatment with 5-aza-2'-d. Cells (60% confluent) were treated with 5 μM 5-aza-2'd for 5 days, with fresh media supplemented with 5-aza-2'd every day. Trichostatin A (TSA, a histone deacetylase inhibitor) was administered at a concentration of 100 nM on the last day of treatment. Cells were harvested and assayed for miR-152 expression by qRT-PCR as previously described [21, 22].

Flow Cytometry Analysis. Cells were harvested 3 days after miR-152 transfection, washed with cold PBS, fixed, and permeabilized with 70% cold ethanol for propidium iodide staining. Cell cycle analysis was performed with a flow cytometer (Accuri, Ann Arbor, MI).

miRNA microarray samples. For miRNA expression profiling, 11 unique cell lines were utilized; specifically, PrEC, RC-77N/E, RC-77T/E, RC-43T/E, RC-43N/E, RC-165N/hTERT, RC-92a/hTERT, RC-170N/hTERT, PC-3, MDA-PCa-2b, and C42B. Microarray hybridizations were performed in triplicate for each line by Ausurgen, Austin TX). In addition, primary tumor samples (n=9) were also processed for microarray hybridization (RC-30T, RC-33T, RC-136T, RC-139T, RC-28T, RC-78T, RC-143T, RC-145T and RC-25T). Clinical characteristics are listed in (supplemental table 4). The protocols for RNA extraction and miRNA microarray hybridizations were performed by an outside vendor (Asuragen, Austin, TX).

Raw data processing from microarrays. For data processing, the bioconductor packages pipelined through R interfaces DaNTE [32] and CARMA [33] were used. Pre-processing and normalization was completed with the Affy package. Two distinct array platforms were used during the course of this project; cell lines were hybridized to one platform and primary samples were hybridized to another. The platforms differ in probe content and replication. Arrays of identical platforms were quantile normalized to minimize inter-array noise [34], using the gcRMA method [35]. Subsequently, replicates were merged into median values for statistical association analyses.

Statistical analysis of normalized data. For all analyses, p-values <0.05 were considered as significant. Initial differential expression was detected using an ANOVA linear model. Of the miRNA genes that showed significant variance, we measured the specific differential expression among categorical groupings; diagnosis, pathological stage, and race. Differential expression associated with categories was measured using a moderated T-test in the limma package of R. Cells lines representing

tumor and uninvolved from the same individuals were grouped into two categories; Malignant or Non-malignant. This grouping also included established laboratory cell lines (PREC, PC3 and C42b). We incorporated multiple hypothesis testing to correct for false positive (type 1 errors) and false negative (type 2 errors). Specifically, we implemented adjusted p-values (Bonferroni) and false discovery rates (FDR) using the Benjamini and Hochberg method [36]. A moderate 10% FDR cutoff was used when applicable. We also used hierarchical clustering analyses to determine groups of differentially expressed miRNAs between specific variables and to isolate specific nodes of probes that shared similar expression trends.

We used non-parametric tests when comparing across array platforms to validate the significance of specific factors in a given variable category. Specifically, a Kruskal-Walis test was used for all variables. In addition, for variables with only 2 levels, a Wilcoxon Rank Sum test was utilized for secondary validation. In cases where strict fold change values were of importance, the fold change between two factor levels was calculated using the ratio of mean expression between the variables over differences in expression between the variables. [$\log(x/y) = \log(x) - \log(y)$].

To determine the ability of significant probe profiles to differentiate samples into relevant categories, we used a partial least squares (PLS) principle component test. Specifically, once probes were found to be significantly differentially expressed, following hierarchical clustering, we identified suitable nodes or combination of probes, for each category. We determined differentially expressed probes based on diagnosis, stage and as the predictors and miRNA expression values as the response. These tests both determined whether the probes statistically associated with a specific variable or had predictive value across independent primary samples not utilized in the initial discovery ANOVA and non-parametric tests. If the largest percent variation could be explained by the variable extracted/tested, and the samples segregated according to proper category variable annotation, the probe set was considered to have significant predictive value for the specified categories. For these analyses, the DAnTE 1.2 program was utilized.

Competing interests

The authors have no conflicting financial interests.

Authors' contributions

ST and CY conceived, designed, performed the experiments, and analyzed the data. MD performed statistical analysis on microarrays and patient samples. HW, TT, and FZ performed experiments. WJ, GZ generated miRNA-mRNA binding constructs. ST, WG and CY wrote the manuscript. All authors have read and approve the manuscript in its final form.

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Figure Legends

Fig. 1**. Heat Map of miRNA microarray**. (a) Hierarchical clustering analyses of miRNAs (left) grouped by non-malignant and malignant status: non-malignant cell lines (red block), malignant cell lines (blue block). (b) PLS plot of AA and CA prostate cell lines grouped by malignancy. (c) Hierarchical clustering analyses of miRNAs (left) grouped by race. AA cell lines (red block), CA cell lines (blue block). miRNAs are ordered according to their cluster determined by p-values using the Kruskal-Walis test. (b) and (d) PLS plot of AA and CA prostate cell lines grouped by race.

Fig. 2**. Multiple miRNA expression pattern after treatment with demethylation agent** (a) LNCaP cells were treated with 5 μM 5-aza-2'd for 4 days alone (+) or with 100 nM TSA for 24 hr (++). Results shown are representative of three independent experiments ± s.e.*P<0.05. (b) PC-3 cells were treated with 5 μM 5-aza-2'd for 4 days alone (+) or with 100 nM TSA for 24 hours (++). Results shown are representative of three independent experiments ± s.e. *P<0.05.

Fig. 3. **The miR-152 promoter contains methylated sequences.** Bisulfite modification was performed on LNCaP, DU-145, and PC-3 cells, and DNA was sequenced to determine the methylation status of the promoter region. Map of miR-152 CpG island sites (EMBL-EBI), and PCR products utilized for bisulfite sequencing.

Fig. 4. **Low miR-152 expression correlation with PCa metastasis.** Samples were sorted by average miR-152 expression and the mean from two probes was normalized with z-score. (a-d) miR-152 expression correlates with metastases, invasion events, and recurrence-free survival in 138 PCa samples (97 primary tumors and 13 metastases) from the Taylor et al. data set. (a) miR-152 levels decrease monotonically from normal, primary tumor, and metastasis, $P < 0.0001$. (b) Intensity in heatmap, with red corresponding to high and blue to low expression. (c) Number of metastatic events separated by high,

intermediate, and low miR-152 expression**.** (d) Low miR-152 expression correlated with decrease in probability of biochemical recurrence free survival p=0.0004 (log-rank test).

Fig 5. **miR-152 expression in AA and CA matched normal tumor cohort**. (a) qRT-PCR of miR-152 expression in normal-tumor paired prostate tissue samples. All samples were normalized to the adjacent normal samples. All values under value one represent down-regulation of miR-152. Note: 67% of the combined patient populations displayed a decrease in miR-152 expression. (b) Plot demonstrating the individual miR-152 expression values in AA and CA patients: Note: 50 % of the individual AA patients had significant lower miR-152 expression compared to only 35 % of CA patients. p-values (Benjamini-Hochberg corrected for multiple tests) .

Fig 6. **Restoring miR-152 expression decreases cell proliferation, migration, and invasion**. (a) Proliferation of LNCaP and (b) PC-3 cells that were transfected with either 30 nM of miR-152 mimic or miR-NC (negative control) was measured by MTT for 6 days. Results shown are representative of three independent experiments averaged and normalized to Day 1 ± s.e. *p<0.05. (c) () LNCaP and (d) PC-3 cells were analyzed by flow cytometry after 3 days of miR-152 mimic transfection (30 nM). miR-152 caused G2-M arrest in cell cycle progression in PCa cell lines. Results shown are representative of three independent experiments. (e) Relative cell migration of LNCaP and PC-3 cells was measured utilizing Boyden migration chambers. Results shown are representative of two individual experiments performed in triplicate. (f) LNCaP and PC-3 cells transfected with miR-152 mimic showed a decrease in the number of cells invading through a layer of Matrigel relative to cells treated with miR-NC (negative control). All data presented are the means of three independent experiments ± s.e. *P<0.05.

Fig. 7. **Comparison of miR-152 and DNMT-1 relationship in PCa cell lines** (a) Relative miRNA and mRNA expressions of miR-152 and DNMT1 were determined by qRT-PCR in a panel of prostate cell

lines with increasing aggressiveness. (b) miR-152 or scrambled oligonucleotides as NC (negative control) treatment of LNCaP and PC-3 cells resulted in decreases of DNMT1 at both RNA and protein levels. (c) Dual-luciferase assays were performed for LNCaP and PC-3 cells co-transfected with the firefly luciferase constructs containing the DNMT1 wild-type or Mu 3′-UTR and miR-152 mimics or (NC) negative control. (d) siRNA-DNMT1 treatment resulted in increases in miR-152 expression in LNCaP and PC-3 cells, as determined by qRT-PCR. DNMT1 expression was analyzed by immunoblots utilizing anti-DNMT1 antibody for both LNCaP and PC-3 cells after siR-DNMT1 treatment. For all qRT-PCR experiments, expression was normalized to RNU48 (miRNA) and GAPDH (mRNA) controls.

Supplemental Fig 1. **Race related miRNAs separate primary tumors cells by race.** (a) PLS plot of individual primary prostate cancer cell isolated from AA and CA patients using race related miRNAs identified from AA prostate cell lines.

Supplemental Fig 2. **qRT-PCR validation of miRNA differentially expressed miRNA in PCa cell lines**. (a-e) Relative miRNA expression of miRs determined by qRT-PCR in prostate cancer cell lines with increasing aggressiveness, compared to normal CA PrEC and AA RC-77N/E cells.

Supplemental Fig 2. ***Insilico*** **analysis of CpG islands of race related miRNAs.** (a). Schematic map of the CpG islands in the promoter region of miR-152, miR-410, miR-376, miR-132.

Supplemental Fig. 3. **Validation of additional miR-152 target genes** (a) RT-PCR validation of the custom-designed miRNA microarray for alogithrim-predicted miR-152 target genes (TargetScan) with or without pre-miR-152 treatment in LNCaP and PC-3 cells. (b) Immunoblots showing that SOS-1 and Rictor were decreased in LNCaP and PC-3 cells treated with pre-miR-152 relative to the NC scramble control.

Fig 1

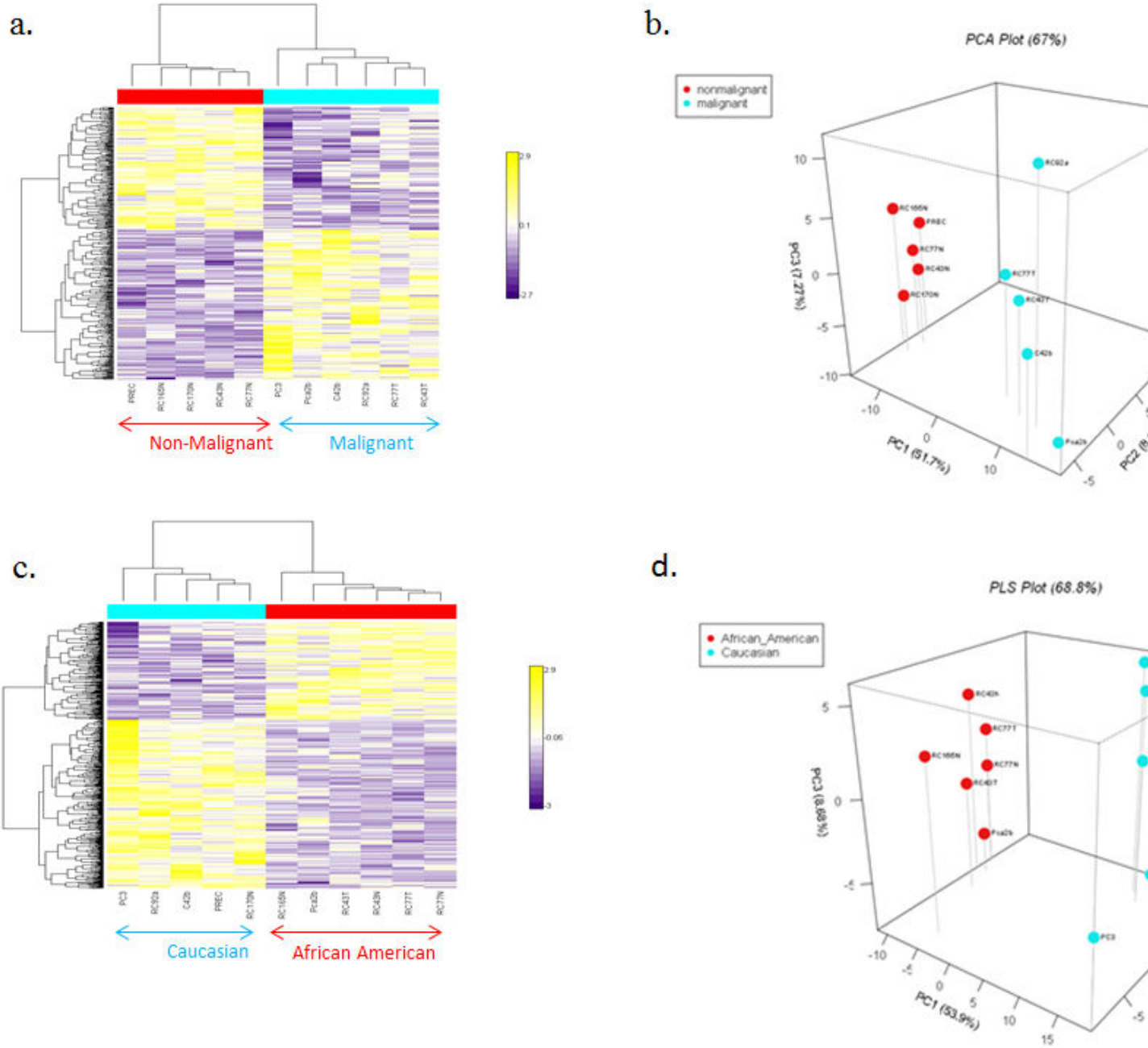
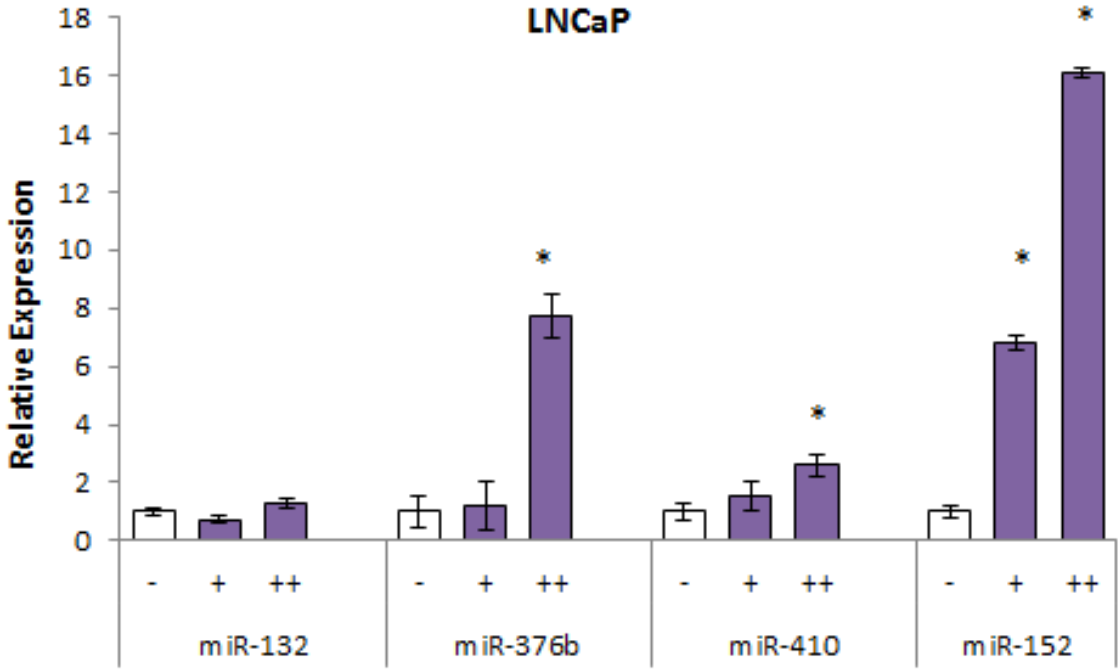


Figure 1

Fig 2

a.



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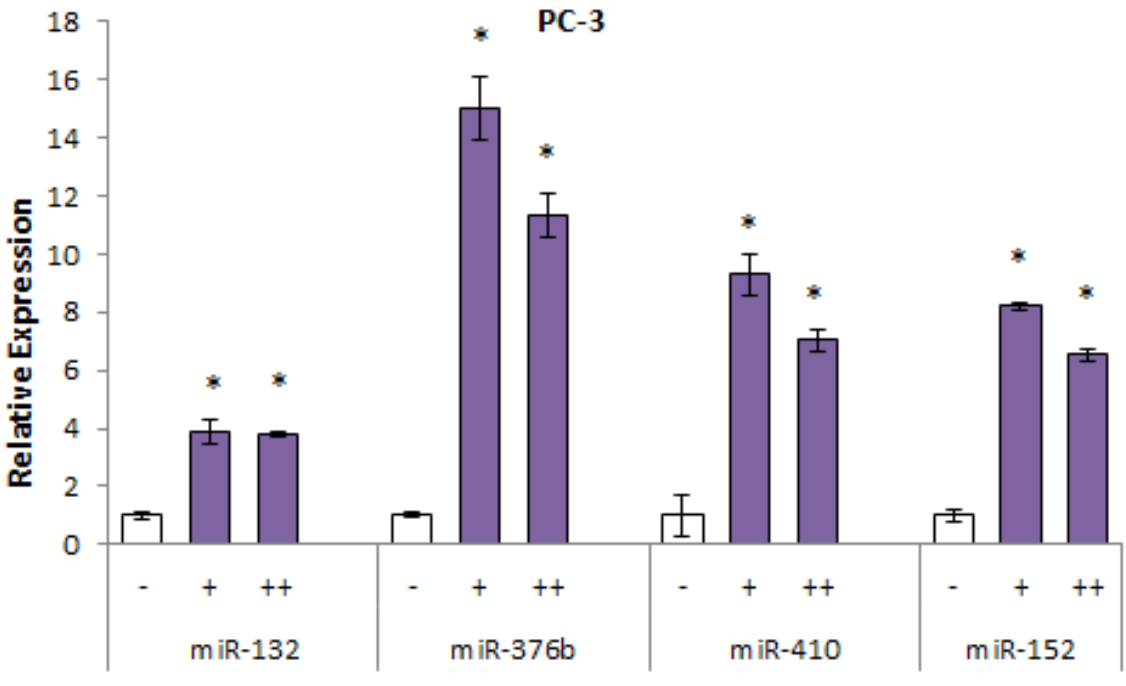


Figure 2

Fig 3

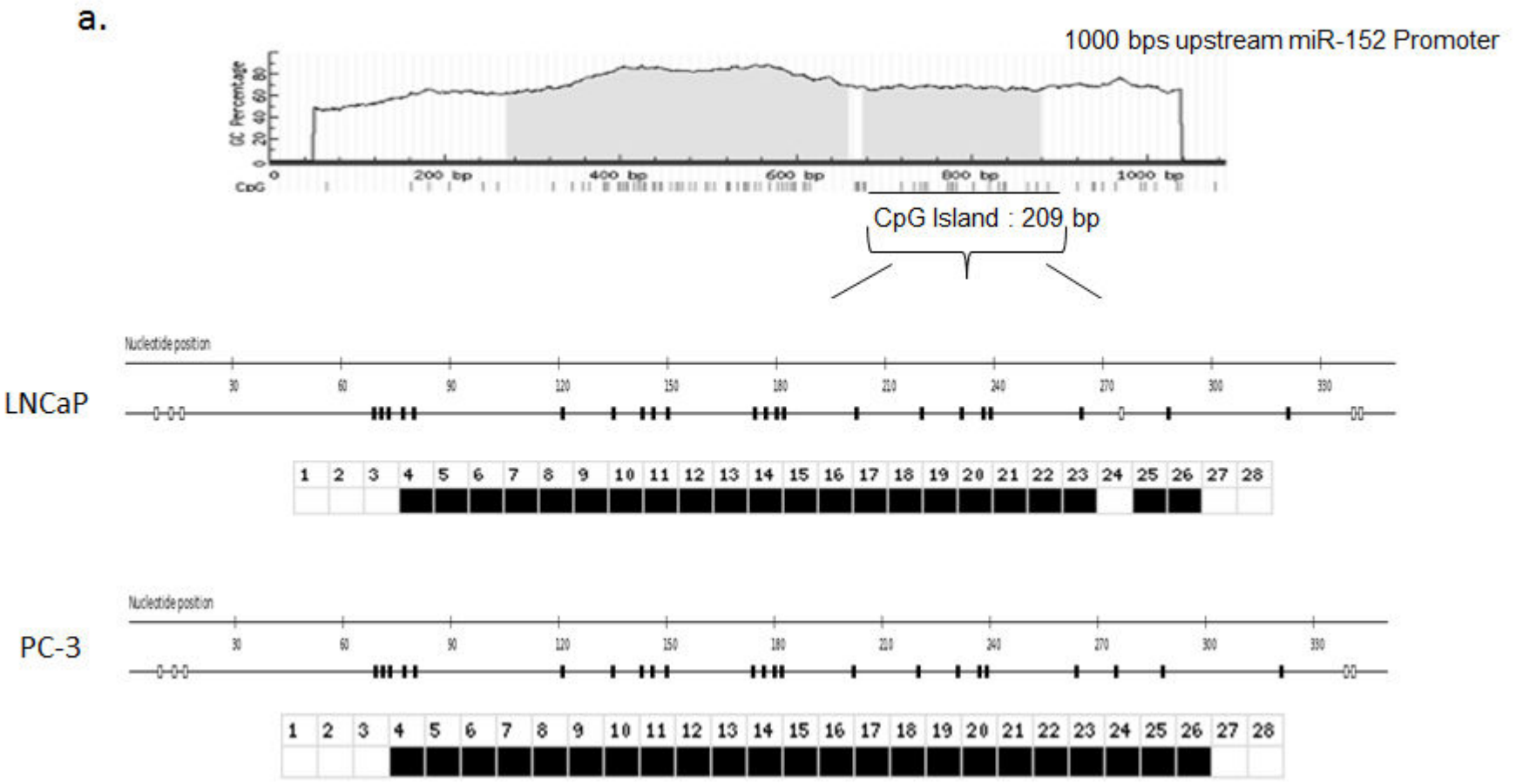
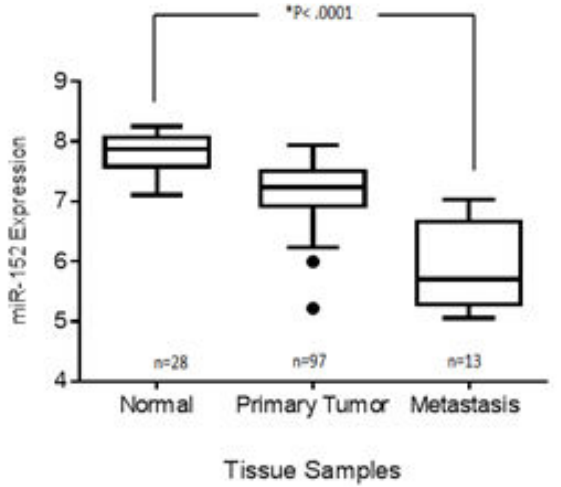


Figure 3

Fig 4

a.



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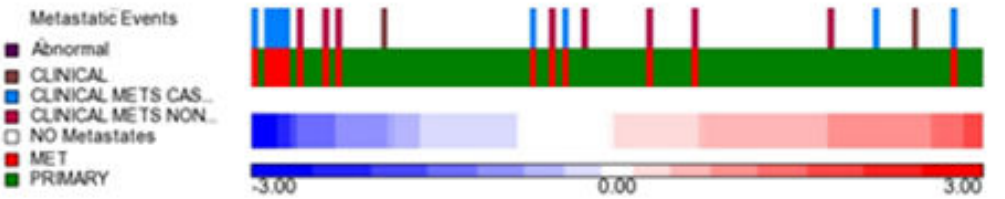

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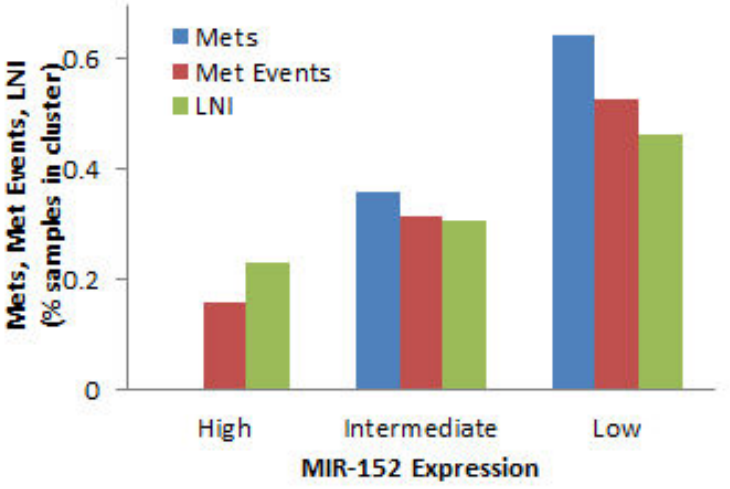

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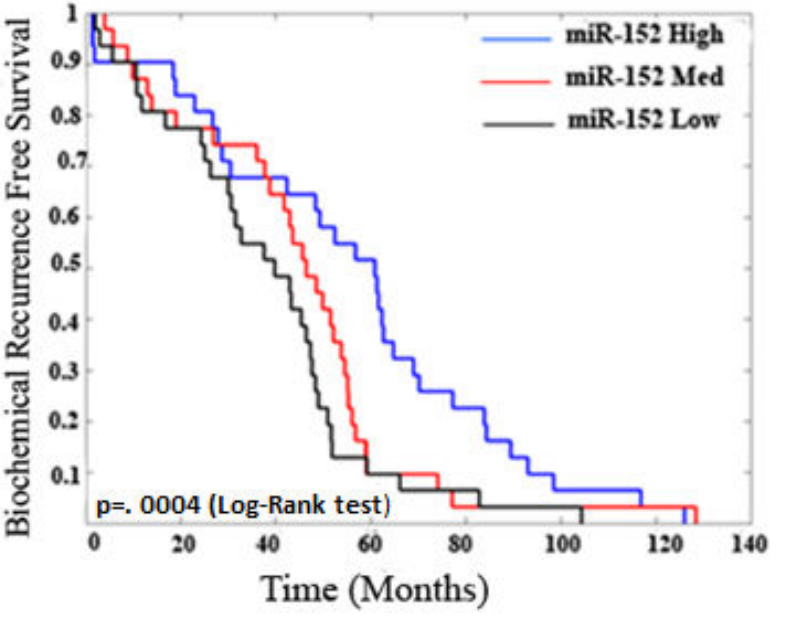
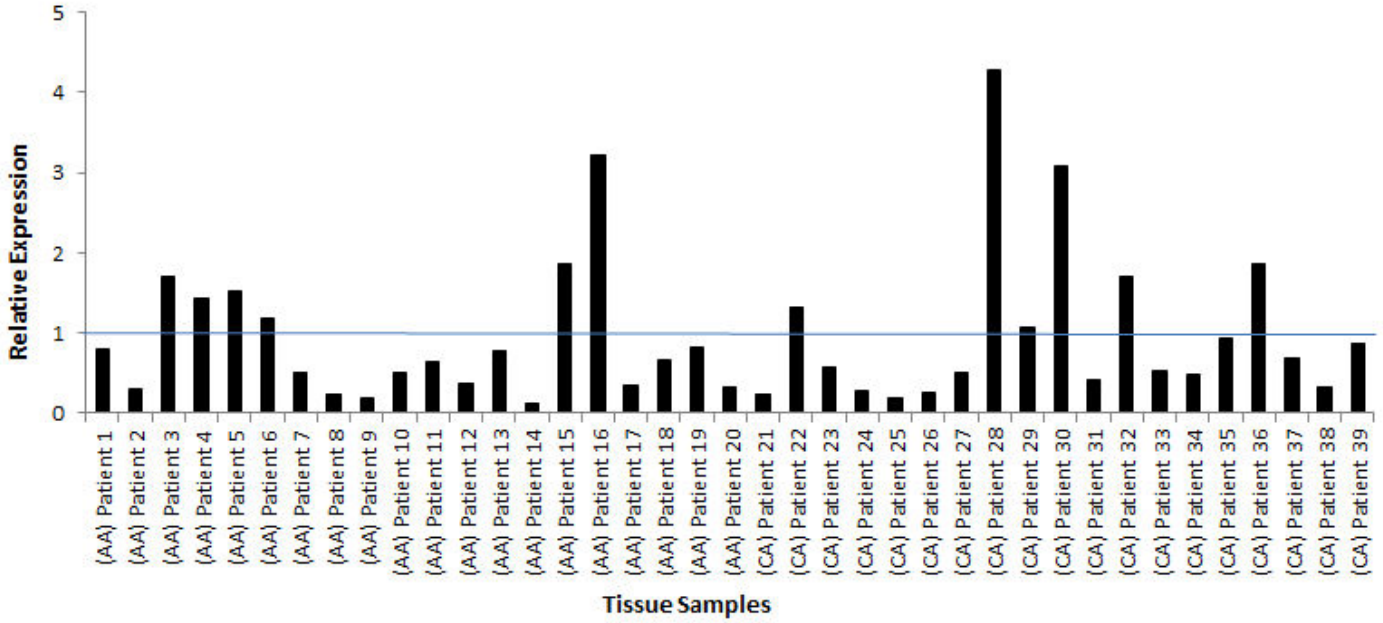


Figure 4

Fig 5

a.



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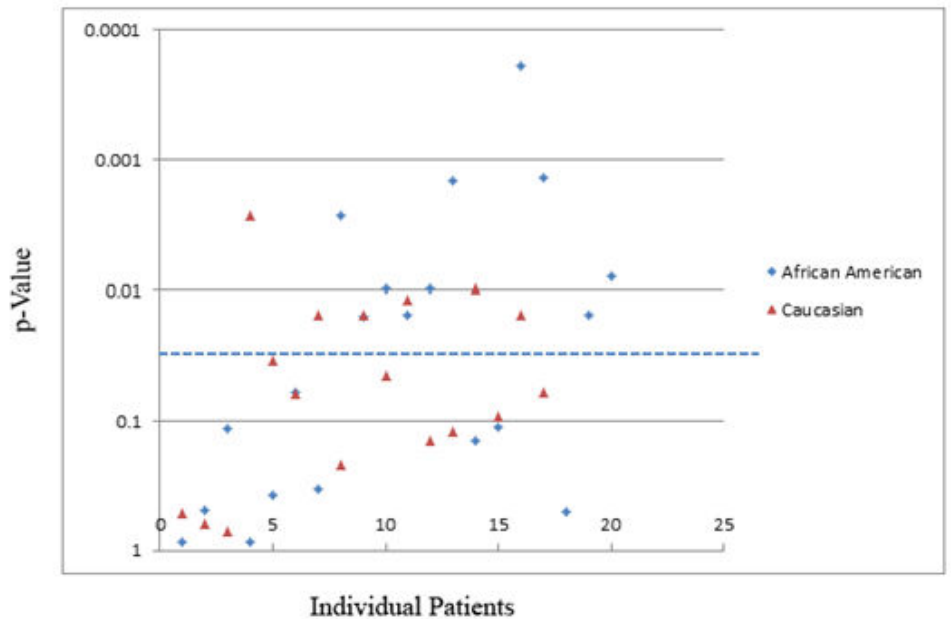

Figure 5

Fig 6

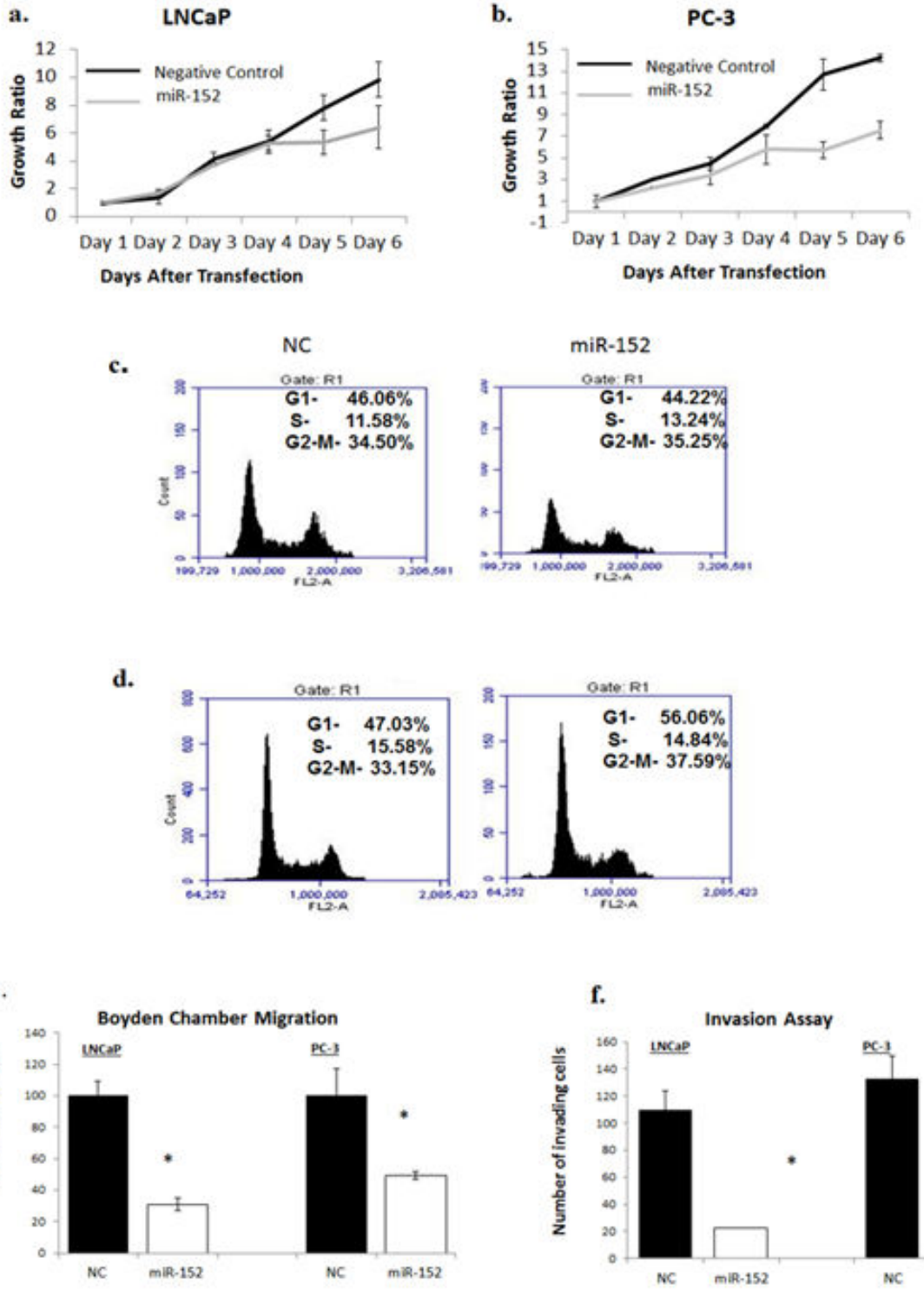

Figure 6

Fig 7

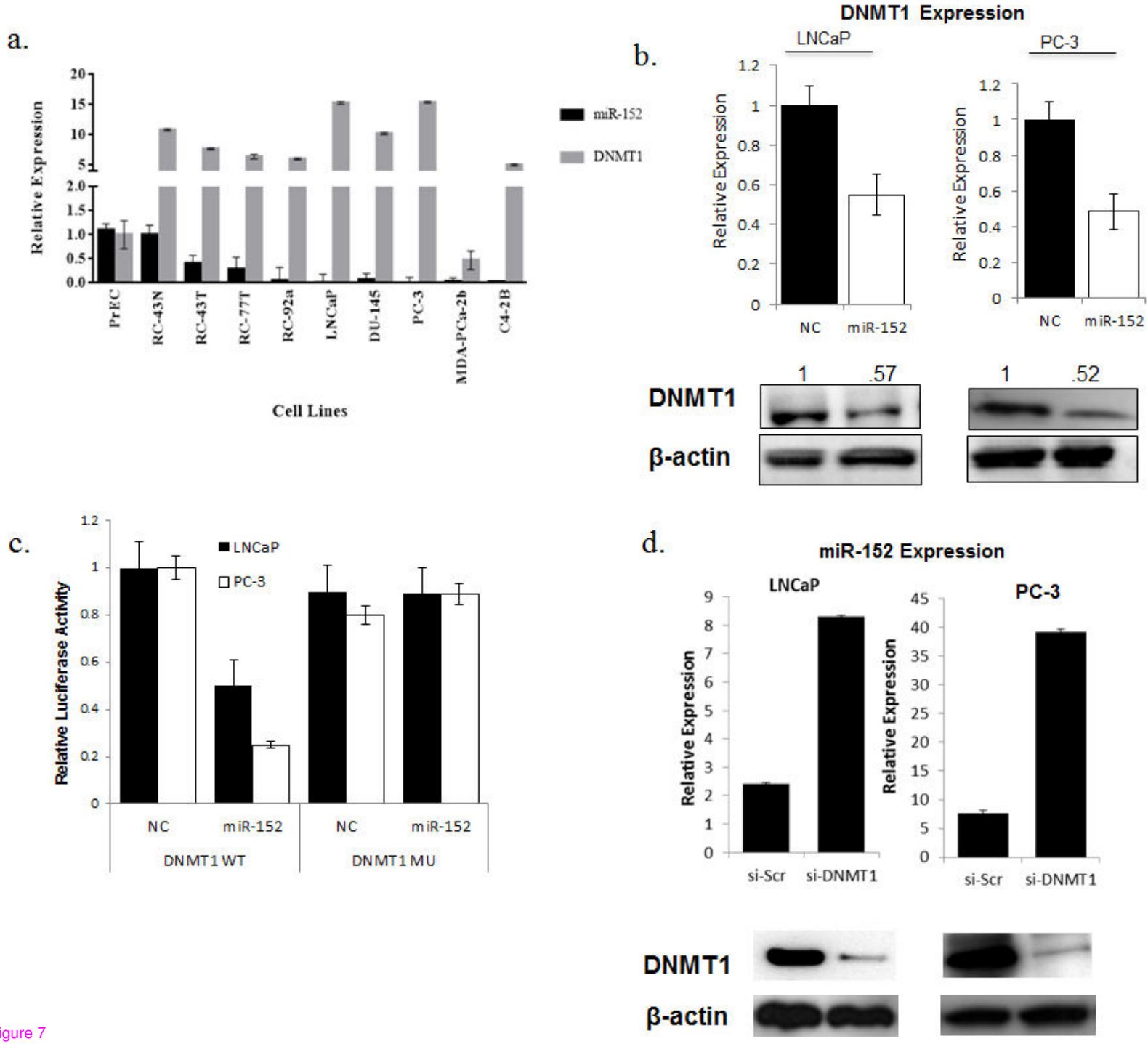

Figure 7

Hierarchical clustering of race related miRNAs	
miRNA	(Adjusted) p-value ↑
hsa-miR-363	3.43e-05
hsa-miR-132	0.000354
hsa-miR-376b	0.00188
hsa-miR-410	0.00291
hsa-miR-152	0.00471
hsa-miR-189	0.00498
hsa-miR110	0.00514
hsa-miR-27b	0.00657
hsa-miR-519c	0.0071
hsa-miR-520h	0.00774
hsa-miR-27a	0.00837

Figure 8

Table 2

Clinical Characteristics of Primary Tumor Paired Prostate Samples

Ethnicity	**African American**	**Caucasian**
Samples	20 (51.3%)	19 (48.7)
Age		
Median	57.5	64
Mean	57.3	60
Standard deviation	6.68	7.3
Min - Max	47-69	48-70
Initial Clinical Stage		
T1	NA	NA
T2	16 (80%)	10 (52.6)
T3	4 (20%)	8 (42.1)
NS	NA	1 (5.3)
Gleason Score		
6	8 (40%)	8 (42.1%)
7	12 (60%)	10 (52.6)
8	NA	1 (5.26)
miR-152 Expression		
Average all samples ±SD	1.6 ±	2.6±

NA – none
SD- Standard Deviation

Figure 9

Additional files provided with this submission:

Additional file 1: Theodore et al Supp Fig 1.tif, 883K
http://www.molecular-cancer.com/imedia/3002316261055537/supp1.tiff
Additional file 2: Theodore et al Supp Fig 2.tif, 2630K
http://www.molecular-cancer.com/imedia/2024527381105553/supp2.tiff
Additional file 3: Theodore et al Supp Fig 3.tif, 1533K
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Additional file 4: Theodore et al Supp Fig 4.tif, 2450K
http://www.molecular-cancer.com/imedia/1748498641055537/supp4.tiff
Additional file 5: Supplemental Table 1.docx, 55K
http://www.molecular-cancer.com/imedia/1859016567105553/supp5.docx
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Additional file 8: Supplemental Table 4.docx, 41K
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Title: Epigenetic field effect markers are indicative of occult high grade prostate cancer.

Authors: Sandra M Gaston, Jihad E Hayek, Gaetan Otto, James Yen, Joseph Bigley, Leander Van Neste, Gary P Kearney; New England Baptist Hospital, Boston, MA; MDxHealth, Liege, Belgium; MDxHealth, Irvine, CA

Background: Epigenetic changes through DNA methylation can serve as biomarkers for the presence of prostate cancer (PCa). Gene methylation in histologically benign tissue adjacent to PCa can provide “field effect" biomarkers to detect cancer missed due to sampling errors. Such errors may also result in underestimates of PCa stage or grade, complicating decisions about Active Surveillance. We previously showed increased field effect methylation in GS 7 over GS 6 subjects in a small patient cohort. Here, established methylation markers of PCa (*GSTP1*, *APC* and *RASSF1*) were tested in a larger cohort to compare histologically benign biopsy cores from patients diagnosed with no cancer, low volume GS 6 cancer and GS 7 cancer. The objective was to confirm that epigenetic field effects may be useful for detecting occult high grade malignancies in diagnostic and prognostic settings.

Methods: DNA was extracted from prostate biopsy tissue prints and used for multiplex methylation-specific PCR (MSP) assays of the 3 genes. All testing was done blinded. Each of 12 tissue cores was tested from a series of 76 biopsy patients: 34 diagnosed with GS 6 PCa, 22 with GS 7 PCa, and 20 no-cancer controls.

Results: In 19 of the 22 GS 7 cases (86%), epigenetic markers were positive for field effects in one or more adjacent benign cores; 12 (54%) were positive for all 3 epigenetic markers. In contrast, only 9 of 34 cases (26%) GS 6 cases were positive for all 3 markers. We again observed that robust marker expression in histologically benign cores from cases diagnosed with low volume GS 6 PCa was associated with higher grade cancer at radical prostatectomy (upgrading). *APC* showed the best performance as a single marker “field effect” test for adjacent high grade PCa.

Conclusions: *GSTP1*, *APC* and/or *RASSF1* gene promoter methylation was observed to be more prevalent in histologically benign cores from biopsy patients diagnosed with GS 7 PCa, as compared with low volume GS 6. This study confirms previous findings in a larger cohort of subjects that these “field effect” biomarkers can be useful for detecting cancer adjacent to histologically negative biopsies and may be indicative of occult aggressive PCa.

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GUEST EDITORIAL
BIOLOGICAL STAIN COMMISSION SYMPOSIUM, 2011

It is primarily the control of transcription and post-transcriptional processing that are critical to the development and progression of sporadic neoplasias

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Control of the growth, progression and dissemination of sporadic neoplasia primarily is by the regulation of transcription, e.g., epigenetic control by methylation, development of somatic mutations in genes, post-transcriptional processing and modulation of translation. While the initial sequences of genes are important, such contributions to risk are relatively modest (Grizzle et al. 2012a,b, Srivastava and Grizzle 2012).

According to Grizzle et al. (2012a), many neoplastic lesions develop in the setting of longstanding continuing damage, inflammation and repair (LOCDIR). The dynamic environment of LOCDIR causes continuing cellular proliferation for which the normal genes, typically involved in embryologic development, normal repair, and responses to damage to tissues, begin to predominate and may become dysregulated. With dysregulation, control of proliferation and cellular death may partially be lost and changes in important regulatory and repair genes, such as microsatellite DNA repair genes, may develop. This becomes a cycle as these changes increase further the chances of mutations and/or dysregulation of genes. For example, mutations in microsatellite repair genes are observed after about one year of ulcerative colitis, but not other inflammatory diseases of the colon (Brentnall et al. 1996, Grizzle et al. 2012a).

Once neoplastic lesions begin to develop, the neoplastic cells hijack and modify normal pathways of repair and embryological growth and development. Changes in the control of these pathways promote survival, growth and progression of neoplastic cells. This process includes increased proliferation and neovascularity of tumors as well as improved survival of neoplastic cells owing to decreased cellular death, e.g., changes in autophagy and/or apoptosis. Important to these processes are autocrine, paracrine and endocrine stimulation provided by signals from microRNAs (miRNAs) and other regulatory molecules including access to these molecules by exosomes and related vesicles whose contents increase proliferation, inhibit apoptosis, increase angiogenesis and decrease immune surveillance of tumors (Zhang and Grizzle 2011, 2013). Thus, in neoplastic development, humans have almost all the genes, regulatory pathways and mechanisms for signal delivery required to support the progression and dissemination of tumors; their selective expression and utilization needs only to be turned on or off for neoplastic processes to proceed.

In general, post-transcriptional processing of genetic information includes pathways and processes by which pre-RNAs produced from DNA are spliced and the resulting mRNA is edited and degraded. Also, factors that control the translation of mRNA into proteins increasingly are understood to be important for post-transcriptional regulation (Blume et al. 2010). In addition, some proteins are modified and degraded after their translation from mRNA and/or are controlled by either their metabolism or other molecular pathways. For example, levels of $p27^{Kip-1}$ are controlled primarily by S-phase kinase associated protein 2, which is involved in the ubiquitination of $p27^{Kip-1}$ and its subsequent degradation (Skp-2). A protein also may be modified after translation, e.g., glycosylation, which may

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change its functions extensively (Probnjak et al. 2003, Moremen et al. 2012, Durand and Seta 2000).

The pre-mRNA produced from transcribing exons and introns of a gene is modified by splicing to remove the mRNA components from introns to produce mRNA or multiple mRNAs by splicing of the exon components. Thus, one gene can produce many different proteins with different functions by alternate splicing of pre-mRNA (Bingle et al. 2002). The production of protein from a specific mRNA is controlled by the rates and degrees of degradation, and by editing of mRNA (Li et al. 2011); a protein cannot be produced from a gene if the transcribed mRNA is degraded before translation. MiRNAs affect the rate and extent of degradation and the translation of mRNA. Effects of miRNAs on translation may result in increased or decreased production of protein (McNally et al. 2013). When an mRNA begins to interact with a ribosome, the translation of the mRNA may be affected at the internal ribosomal entry sites (IRES), which may be modulated by various endogenous proteins, small RNAs and drugs (Blume et al. 2010).

MiRNAs are small RNAs that are produced from areas of the DNA that are not transcribed into mRNA. MiRNAs can be regulated by methylation of their gene control elements and typically bind to the 3′ untranslated areas of mRNA. Their binding to mRNAs is a major regulatory feature that controls the degradation of mRNAs. MiRNAs may suppress oncogenic features (suppressamiRNAs), stimulate oncogenic features (oncomiRNAs) or modulate the ability of neoplastic cells to metastasize (metastamiRNAs) (Cho 2007, Esquela-Kerscher and Slack 2006, Hurst et al. 2009). More details concerning how miRNAs affect post-transcriptional regulation of the development, progression and metastasis of neoplasia are described in the articles of this special issue.

Analysis of miRNA in tissues presents multiple challenges. One challenge for studying the involvement of miRNAs in various cancers is the choice of control tissue. Frequently, when miRNAs are studied in a specific type of neoplasia, control tissue with which malignant lesions are compared may not represent the tissue from which the tumors developed. This is a challenge especially when the cells of origin of a tumor are controversial, such as ovarian papillary serous carcinomas, e.g., ovarian surface epithelial cells vs. cells of the fallopian tubes vs. cells of ovarian microcysts. Similarly, when controls are selected for colorectal cancer, cells of the epithelium of normal or uninvolved colorectum should be the control rather than the cells from the entire wall of the colorectum, which includes smooth muscle. In addition, areas of the colorectum vary biologically owing to differences in embryology, vascular supply and drainage, function, therapy, i.e., colon vs. rectum, as well as molecular features (Manne et al. 2012). For example, tumors with mutations in microsatellite DNA repair genes usually occur in the proximal colon. Thus, the choice of controls for colorectal cancers may be complex.

Another challenge for analyzing the association of miRNAs or other molecules with specific cancers is the mixture in a tumor of non-neoplastic cells, which are prominent components of some tumors (e.g., lymphocytes) as well as some control tissues (e.g., colonic mucosa). Because all cells in tumors, both normal and malignant, produce miRNAs, analysis of miRNAs, may require exclusion or reduction of non-malignant cells in the specimen that is analyzed. This may be a greater problem with control tissues in which inflammatory cells cannot be removed without laser capture microdissection (Fig. 1).

A third challenge is to determine and select an optimal sample in which to measure miRNAs. For example, in some cancers and controls, such as those from pancreas, enzymes may destroy miRNAs rapidly. Similarly, because they are small molecules, miRNAs may be lost by diffusion from tissue samples during processing steps such as staining frozen sections to select tumor cells for microdissection, or when paraffin or frozen sections used for extraction of RNA are too thin (e.g., <10 μm). Frequently, investigators use tissue in which miRNA can be detected rather than determining the optimal type of tissue for miRNA measurements. Continuing questions include whether miRNAs should be measured in fresh, frozen, fixed or fixed and paraffin embedded tissues and whether the optimal specimen varies with the specific type of tissue and/or with specific miRNAs. Such issues are evaluated inadequately in most studies.

Even with these challenges, miRNAs have been associated with the diagnosis of specific neoplastic lesions, and with prognosis, clinical outcomes and responses following specific therapies (McNally et al. 2013, Bovell et al. 2013, Jones et al. 2013). Of increasing interest is the use of miRNAs for early diagnosis of cancers including selective measurement of miRNAs within exosomes and related vesicles. For example, miRNAs in exosomes have been used for early detection of multiple cancers (Zhang and Grizzle 2011, 2013). In addition, engineered short segments of RNA (antagomiRNAs) now are being used to target specific miRNAs for potential therapeutic uses (Krützfeldt et al. 2005).

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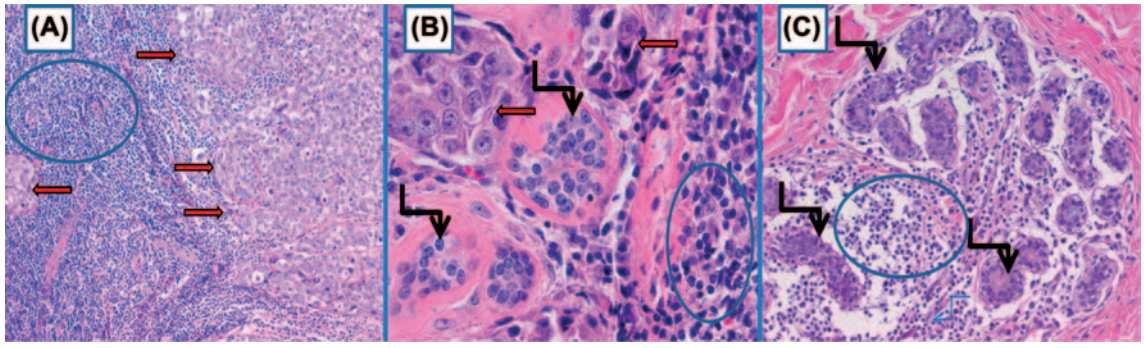

Fig. 1. Samples of tumors and uninvolved or normal tissue (i.e., controls) frequently are composed of heterogeneous cell types. MiRNAs can be secreted by any of these cell types and thus may complicate interpretation of measurements of miRNA. A) Malignant breast cells (red arrows) adjacent to lymphocytes (blue circle). Original magnification × 100. B) Foci of malignant breast cells (red arrows) intermixed with uninvolved breast cells (black arrows) and lymphocytes (blue circle). Original magnification × 400. C) Uninvolved breast cells (black arrow) surrounded primarily by lymphocytes (blue circle). Original magnification × 400.

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focus is on the effects of miRNA in the post-transcriptional regulation of mRNAs and how miRNAs affect neoplasia.

We begin with the review by McNally et al. (2013), which describes the post-transcriptional regulation of genetic information and includes the basics of miRNAs from their synthesis to their involvement in the development and progression of neoplasia. Specifically, how miRNAs are named/designated and delivered (e.g., by exosomes), how miRNAs modulate mRNAs, and the importance of miRNAs in cancer are reviewed briefly. Also, the effects of other non-coding small RNAs on cancer and cancer research are described.

The article by Dr. Ajay Singh and co-investigators reviews current and future methods by which miRNAs can be measured and analyzed. This article also discusses briefly the importance of miRNAs in cancer. Methods discussed include real time, reverse transcriptase, quantitative polymerase chain reaction (RT^2-Q-PCR) including modifications of RT^2-Q-PCR to permit rapid, concomitant screening of most miRNAs (Srivastava et al. 2013).

The article by Dr. Upender Manne's group describes how miRNAs affect selected cancers and how they can be used as biomarkers for diagnosis or for determining prognosis. They describe studies in which miRNA has been measured not only in blood, but also in urine and saliva. Also discussed are how miRNAs affect chemoresistance of various neoplastic lesions and how miRNAs may be important for medical advances (Bovell et al. 2013).

The article by Dr. Clayton Yates' group reviews the effects of post-transcriptional regulation of prostate cancer (PCa) by miRNAs. Differences in prostatic cancer (PCa) in African Americans vs. Caucasian Americans are described. The actions of miRNAs in PCa as oncogenes (oncomiRNAs), modulators of metastases (metastamiRNAs), and as suppressor genes are described with special emphasis on post-transcriptional regulation that leads to more aggressive forms of PCa. Also, these investigators review previous reports of differential expression of miRNA-1b-1, miRNA-26a, miRNA-30c-1, miRNA-151, miRNA-219, and miRNA-301 in prostatic adenocarcinomas of African-Americans and report confirmation of differential expression of miRNA-26a in prostate cancer cell lines derived from African Americans compared to cell lines of prostate cancer derived from Caucasian Americans (Jones et al. 2013).

The study from the laboratory of Dr. Lacey McNally reports the differential expression of miRNAs between ovarian papillary serous (OSC) carcinoma cells in ascites and matched metastatic lesions in the omentum. In cells isolated from ascites, the expressions of miRNA-21, miRNA-31, miRNA-100, miRNA-141, miRNA-214, and miRNA-483 were increased and the expressions of miRNA-10a, miRNA-146, miRNA-183, miRNA-196a, miRNA-382, and miRNA -501 were decreased compared to matched omental metastases. They also describe differences in chemoresistance of ovarian papillary serous carcinoma and associated changes in miRNA-21 and miRNA-214. They noted that expressions of miRNA-21 and miRNA-214 were correlated with loss of PTEN, a target of miRNA-21, and resistance to platinum therapy in OSC and note that this observation has been reported for other types of cancers (Frederick et al. 2013).

Another article by Dr. McNally's group reports that the regulation of the expression of insulin-like growth factor 1 receptor (IGF1-R) is regulated by miRNA-100 in pancreatic cancer. MiRNA-100 and miRNA-138 were expressed differentially in cell lines

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of pancreatic cancer that have the potential to metastasize compared to pancreatic cancer cell lines that do not metastasize. Similarly, IGF1-R was correlated with differential expression in potentially metastatic pancreatic cancer cell lines and miRNA-100 was found to regulate IGF1-R in these cells (Huang et al. 2013).

We hope this issue of *Biotechnic & Histochemistry* will be useful to readers who are not well versed in this important area of biology as well as investigators who are just beginning to work with miRNAs.

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Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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